Gene and Cell Therapy

Therapeutic Mechanisms and Strategies
Second Edition, Revised and Expanded

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To our friends and colleagues
Ralph Dornburg, Jeffrey Isner, Danilo D. Lasic, and Michael Strauss

Foreword: The Past Recalls the Future

In the three short years since the first edition of this seminal volume was published, myriad events in gene therapy have occurred. It is far beyond the scope of this short introduction to review events in detail, but several serve as pivotal reminders that within the goal of promoting and enhancing the public health to serve the disenfranchised, progress depends on the highest ethical framework for clinical investigation and the most rigor that medicine and science can offer.

Jesse Gelsinger's death in 1999 led to a reexamination by the Food and Drug Administration (FDA) of the state of gene therapy clinical trials conducted in the United States. In an unprecedented approach, a random sampling of all gene therapy investigative new drugs led to FDA bioresearch monitoring (BIMO) inspections of 70 clinical sites. Results of that intensive effort were reported at an FDA Biological Response Modifiers Advisory Committee Meeting in April 2001 (http://www.fda.gov/ohrms/dockets/ac/01/briefing/3739b1.htm). An important conclusion was that ''The random surveillance inspections of Phase 1 and 2 gene therapy clinical trials indicate that the trials were being properly conducted with fewer deviations than found in Phase 3 studies.'' Although reassuring, there were common deficiencies, such as failure to follow the protocol and inadequate consent form, that need to be corrected on a continual basis in order to ensure that meaningful information can be gathered from clinical trials.

Another issue raised by Jesse's death was that for adenoviral vector gene therapy studies, no adequate means existed in 1999 to compare dosing across clinical studies. At a December 1999 Recombinant DNA Advisory Committee meeting, a challenge was made to create a reference material, and the response by the community bears remarking. An international task group along with the FDA and the Williamsburg Bioprocessing Foundation undertook a unique proposal (see details under Reference Materials Projects at http://www.wilbio.com). Pro bono bids were solicited for various aspects of creating a reference material, such as production of a seed lot, characterization and testing, vialing, and shipping. As of August 23, 2002, the Adenoviral Reference Material is available for purchase from the American Type Culture Collection (ATCC), with full characterization data. The availability of this material allows production of more consistent, safer, quality adenoviral vectors and comparability between preclinical and clinical studies, and greatly aids the development of regulatory policy.

A final remarkable event concerns the poignant clinical study of the use of murine retroviral vector–based gene therapy of X-linked severe combined immunodeficiency disease (XSCID), conducted by Dr. Alain Fischer and colleagues in France. Nine of ten children treated in that trial have had successful immune reconstitution that appears to be superior to that seen with conventional bone marrow transplantation. In August and December 2002, Dr. Fischer communicated to gene therapy investigators and regulatory agencies that two of the nine successfully treated children have developed T cell leukemia-like syndromes. Both adverse events appear to be caused, in whole or in part, by insertion of the therapeutic gene in or near a proto-oncogene locus called LMO-2. A number of select committees worldwide have and are continuing to investigate these findings, with the emerging consensus being that the gene therapy works in XSCID, but, because of the adverse events, gene therapy should be used only when no available alternatives exist.

Several items bear further discussion. First, Dr. Fischer and colleagues must be credited and thanked for having the courage to communicate these adverse-event findings, even before the molecular cause was known. Without this open communication, progress in therapies will be slowed and in jeopardy. Second, it must be taken in context that most clinical trials fail in achieving even preliminary data suggesting therapeutic benefit. Here we have a study in which the therapy clearly benefits but where there are now known adverse events associated with the therapy. Third, it is rare that scientific methodology allows us to determine

rapidly that the therapy is the direct cause of the adverse events. The challenge to all of us is, given this knowledge of benefits and risks, how can we preserve that which is desired—the cure of X-SCID—and mitigate and/or eliminate the adverse events?

I am once again honored by the request of the publisher and editors to provide this brief introduction. The authors are representative of the large arena of gene therapy participants, and as such they and others forward the field in a professional, collegial, and rigorous manner. It is my hope that the authors continue to consider the FDA to be a partner in these efforts.

In conclusion, from the previous edition: ''And to end this brief foreword, let us be sober and focus on the word 'reality'. At this writing, no gene therapy product has been approved by the FDA as safe and effective for any disease. It is the hope that that will happen in the future. How near that future is depends on all of us, the public, the FDA, the NIH, the industry, to work, debate, negotiate, and create the new millennium.''

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Preface

Since the first publication of this book in 2000, gene therapy clinical trials have yielded some remarkable successes and some disappointing failures. In many research laboratories, valuable insights have been gained that will enable us to overcome many of the difficulties encountered to date and to move gene therapeutics toward routine use in the clinic. As with any new therapy, such as heart transplantation, many failures must be expected before treatment becomes routine. Long-term success requires that we rationally assess each failure and determine what additional knowledge is required to solve each problem we encounter. Once again, the authors of this second edition have provided thoughtful insights into the challenges that must be met for specific applications of gene and cell therapies.

The ongoing development of gene therapy draws from many disciplines, including virology, molecular biology, medicine, genetics, immunology, biochemistry, physiology, chemistry, biophysics, molecular imaging, cellular biology, microbiology, pharmacology, and toxicology. This book includes topics in each of these areas and has been planned to facilitate its ongoing use as a textbook for classes in gene therapy and gene transfer. The chapters are written so that readers from all backgrounds, including students, physicians, scientists, and other interested persons, can understand the current status of gene therapy and how these diverse disciplines contribute to this field. Several contributors have broadened their introductions to facilitate use of this edition as an educational textbook and have contributed information to provide context for their focused research efforts. Hopefully, this volume will provide all readers with a broad knowledge of all aspects and tools available in the evolving field of gene therapy.

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1

Retroviral Vectors for Gene Therapy

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I. INTRODUCTION

Gene therapy aims to treat both genetic and infectious diseases by the introduction of new genetic material into the appropriate cells in the body (1). In the simplest case of a defective gene causing disease, addition of the new gene will restore function. Alternatively the new genetic material can be designed to selectively kill a tumor cell, to induce an immune response, or to protectively ''immunize'' a cell against an incoming pathogen. Nontherapeutic uses of gene therapy include gene marking, which has proved especially useful in identifying the sources of recurring malignancies in autologous bone marrow transplant patients. These potential applications of gene therapy are described in Table 1.

The first approved gene therapy clinical protocol began in September 1990, using retroviral vectors to introduce copies of the adenosine deaminase (ADA) gene into T cells from a patient with ADA deficiency (2,3). In 2002, over 600 clinical protocols have been approved worldwide, incorporating over 3500 patients. Most of these trials have used viral vectors, with the largest number (34% of trials, 50% of total patients) involving retroviral vectors. This chapter explores the features of retroviral vectors that make them so versatile for gene therapy and highlights both their current limitations and potential improvements.

II. RETROVIRAL VECTORS

Retroviruses can efficiently carry out gene transfer to many cell types, where they stably integrate their genomes into the host cell chromosome. Gene transfer vectors derived from retroviruses therefore provide the possibility of long-term gene expression. To date, the most common vectors used in clinical gene therapy protocols have been based on the murine leukemia virus (MuLV), and various packaging systems to enclose the vector genome within viral particles have been developed [reviewed in (4)]. The vectors themselves have all the viral genes removed, are fully replication defective, and can accept up to approximately 6–8 kilobase (kb) of exogenous DNA.

A. Retroviral Life Cycle

Fundamental to the utility of retroviral vectors are the particular characteristics of the retroviral life cycle, illustrated in Fig. 1. The initial steps of the life cycle, from binding of the virus to a target cell through integration of its genetic material into that cell's genome, do not require the *de novo* synthesis of any viral proteins. Accordingly, a retroviral particle can be used as a vector to deliver genetic material without the requirement for any viral protein synthesis or infectious particle production.

The retroviral genome codes for 3 basic polyproteins (Fig. 2A), produced by alternate splicing of the RNA transcript, which are further processed into their component proteins. The *gag* gene products produce the protein core of the viral particle, which encapsidates 2 copies of the linear RNA genome. Also associated with the viral core are the products of the *pol* gene, the enzymes involved in particle maturation (protease) and DNA metabolism (reverse transcriptase and integrase). The *env* gene product is a glycoprotein that is anchored in the plasma membrane of the host cell. It is therefore incorporated into the lipid envelope that surrounds the retroviral particle as a result of its budding from a host cell. The budding process does not kill the host cell, which allows for the establishment of stable producer cell lines that continuously release retroviral vector particles.

Table 1 Gene Therapy Applications

1. Gene replacement/augmentation
   Especially suited to single gene defects and discrete populations of target cells (e.g., ADA deficiency, cystic fibrosis)
2. Suicide/toxic genes
   To eliminate certain cells (e.g., HSV thymidine kinase for cancer cells)
3. Protective genes
   Expression of the gene product renders the cell resistant to viral attack (e.g., intracellular antibodies, antisense constructs for HIV gene therapy)
4. Immune stimulation
   To stimulate the host's immune system, in particular, to cancer cells (e.g., HLA genes, immune stipulatory cytokines)
5. Cell marking
   For autologous bone marrow transplantation in cancer therapy (e.g., neomycin)

A retrovirus binds to a new host cell by virtue of the interaction of the Env glycoprotein with an appropriate cellular receptor. This interaction triggers a series of events that ultimately lead to the fusion of the lipid envelope surrounding the virus with the target cell membrane. Entry of the retroviral core into the cell allows the reverse transcriptase enzyme to copy the viral RNA genome into a double-stranded DNA provirus, which is then randomly inserted into a host chromosome through the action of the integrase protein. Certain sequences in the RNA genome are essential for packaging, reverse transcription, and integration to occur, and are highlighted in Fig. 2B.

B. Basic Components of Retroviral Vectors

The simplest type of vector system comprises 2 components: a packaging construct that provides all the viral proteins in *trans* but is not itself packaged because of a deletion in the packaging signal (ψ) at the 5′ end of the genome, and a vector genome that codes for no viral proteins but retains all the necessary RNA regions for packaging, reverse transcription, and integration (5). This basic principle of vector design is illustrated in Fig. 3. The gene to be delivered is cloned into the vector genome construct, and in the simplest arrangement uses the 5′ long terminal repeat (LTR) promoter to drive its subsequent expression. When both the vector and packaging constructs are present in a producer cell, retroviral vector particles are released that are capable of delivering the vector genome with its inserted gene. This process of gene delivery is referred to as transduction. Such strategies have been applied to derive vector systems from several different types of retroviruses, including the murine and avian oncoviruses (5,6), human and nonhuman lentiviruses (see Section V.B), and foamy retroviruses (7).

C. Improvements in Vector Design

The basic arrangement described above is functional but unsatisfactory in several ways. In particular, the considerable sequence overlap that exists between the vector and packaging components means that there is a very high risk of recombination occurring that could create an infectious replication-competent retrovirus (RCR) (8). Overlap occurs because extensive sequences of the *gag* gene are retained in the vector to enhance the efficiency of packaging (9), although Gag protein expression itself is prevented by mutation. In addition, early vector systems retained both the 5′ and 3′ LTRs in the packaging construct to provide promoter and polyadenylation sequences. Finally, because most of the early MuLV-based packaging cell lines were established in murine NIH 3T3 cells, the possibility also exists for RCR generation through recombination between vector components and endogenous MuLV-like sequences present in the mouse genome.

To minimize the risk of RCR production, an early improvement in vector design was to split the packaging component, placing the Gag-Pol and Env genes on 2 separate plasmids that could be introduced separately into the packaging cell (10,11) (Fig. 4A). The risk of recombination has been further reduced by the use of heterologous Env proteins that have no homology with the parental virus but are able to be incorporated into the viral particle (a process referred to as pseudotyping), and the use of nonmurine producer cell lines. Finally, it has now been shown for MuLV vectors that the *gag* sequences can be removed from the vector genome without significant loss of packaging efficiency (12).

The problem of the LTR overlap that exists between the vector components has been solved through the use of heterologous promoters and polyadenylation signals in the packaging constructs. This can also have the advantage of enhancing titer (13) because the MuLV LTR promoter will not always drive high-level gene expression in nonmurine producer cell lines. In the vector itself, the LTR sequences can also be significantly deleted. Heterologous promoters, frequently the cytomegalovirus (CMV) immediate-early promoter, have been used to replace the 5′ U3 promoter (13), which is possible because the U3 sequences in the retroviral vector are derived from the 3′ LTR. Even the 3′ U3 sequences can be significantly deleted, as is the case with self-inactivating (SIN) vectors, as long as the sequences necessary for recognition by the integrase protein are retained (14). These features are summarized in Fig. 4B.

Improvements have also been made in the titer [number of colony-forming units (cfu) per milliliter] achieved by retroviral vectors. For example, in stable producer cell lines, vector production can be maximized by linking expression of the Gag-Pol and Env components to selectable markers to facilitate screening for high titer producer cells (15). Titers have also been boosted through the development of transient expression systems, which are capable of producing very high titers during a brief and very active burst of activity in a transiently transfected cell. Typically, these systems rely on the use of a highly transfectable cell line such as 293T cells, com-

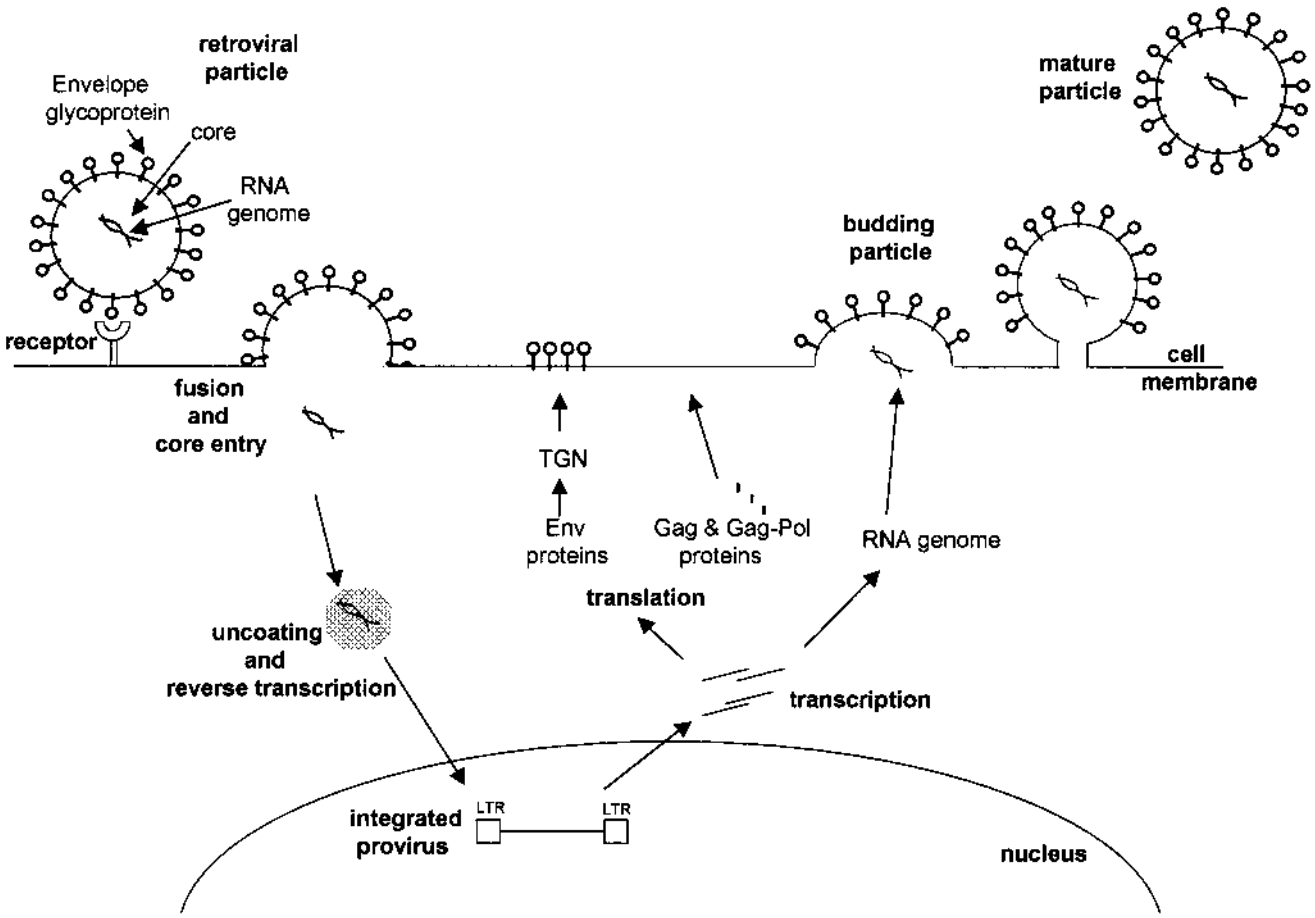

Figure 1 Retroviral life cycle. Retroviral infection is initiated by binding of the envelope glycoprotein embedded in the outer lipid membrane of the retrovirus to a specific cell surface receptor. This interaction triggers fusion between the viral and host cell membranes and releases the viral core into the cytoplasm of the cell. The viral RNA genome is transcribed into a DNA copy by the viral reverse transcriptase protein and is integrated into the host cell chromosome by the action of the integrase protein. The inserted provirus is flanked by complete copies of the LTR sequence, resulting from the reverse transcription process. The 5′ LTR drives transcription of the retroviral genome, which gives rise to RNAs that code for the viral proteins (Gag, Pol, and Env) as well as the viral genome. Gag and Gag-Pol proteins assemble as viral core particles at the plasma membrane and package the viral RNA genome. The particles bud from the surface of the cell, taking with them a lipid envelope derived from the host plasma membrane containing the Env glycoprotein. TGN, trans-Golgi network.

bined with strategies to maximize the production of the individual components through the use of the strong CMV promoter and treatment with the transcription enhancer sodium butyrate (13). We have also shown that titers can be further improved by the inclusion of the adenovirus VAI gene to enhance translation (Lin and Cannon, unpublished data). The combination of these various approaches enables the routine production of vector supernatants in the laboratory with titers in excess of 10^7 cfu/mL. Whether such transient production systems will ever be useful for large-scale production is uncertain because of potential difficulties in the scale-up and vector characterization procedures.

Although currently preferred for large-scale vector production, the use of stable producer cell lines precludes the use of cytotoxic components. These can include both the therapeutic gene product itself as well as components of the vector system. A notable example is the vesicular stomatitis virus G protein (VSV-G), which is an extremely useful fusion protein for producing pseudotyped retroviral vectors with a very broad host range (16), but is unfortunately very toxic to its host cell. One way around this problem is to use transient systems as described above (17), but an alternate strategy is to regulate its expression through use of an inducible promoter. In particular, the tetracycline-regulated Tet system (18) has been exploited to regulate VSV-G (19), where expression is suppressed in the producer cell line by the addition of tetracycline to the culture and activated by removal of the antibiotic at an appropriate time before harvesting the vectors. In this way,

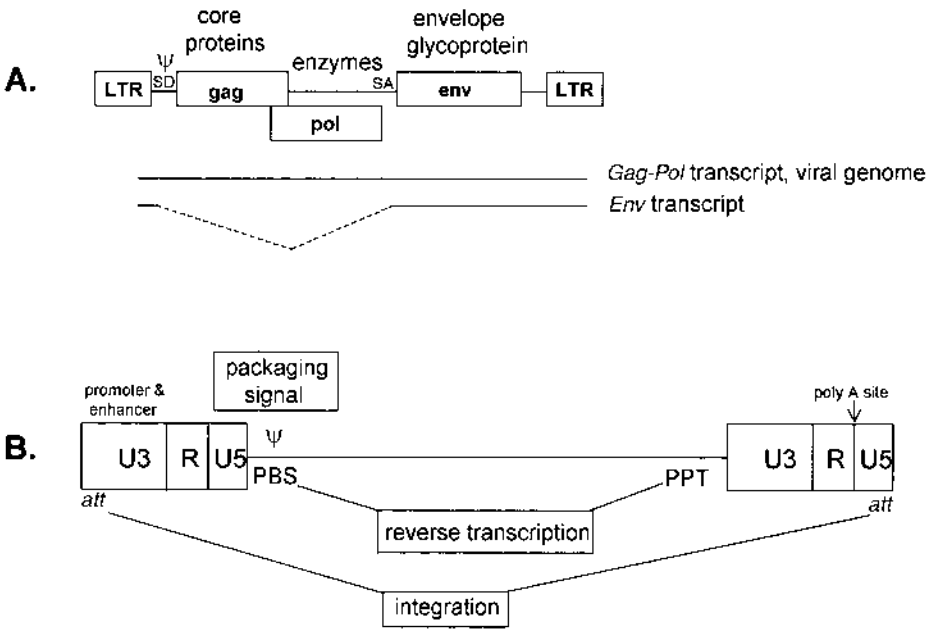

Figure 2 (A) Retroviral proteins. The LTR sequences contain promoter (5′) and polyadenylation (3′) sequences and produce full-length and spliced transcripts. These code for 3 major polyproteins; Gag and Gag-Pol are translated from the full-length transcript and Env is translated from the spliced transcript. The full-length transcript also serves as the RNA genome. SD, splice donor; SA, splice acceptor. (B) RNA sequences. The LTRs consist of three regions, designated U3, R, and U5. The promoter and enhancer sequences are active in the 5′ LTR only and are located in the U3 region, whereas the polyadenylation (poly A) site in the 3′ LTR defines the R/U5 boundary. The primer binding site (PBS) and polypurine tract (PPT) are important for the process of reverse transcription, whereas the *att* sequences at the ends of the LTRs are necessary for integration. At the 5′ region of the genome is a packaging signal (ψ) that is necessary for the incorporation of the genome into viral particles.

the cells can be grown to an optimum density before this toxic fusion protein is expressed. However, although such cell lines are appropriate for laboratory-scale preparation, it is not clear if such a system will be sufficiently stable for industrial production.

As stated in the introduction, retroviral vectors are the most common gene delivery system to have been used in human gene therapy protocols. This is partly for historical reasons; vectors derived from MuLV were the first real vector system to be established and a relatively large amount of information about the performance of such vectors in patients is available. However, their most characteristic feature, the ability to integrate into target cells, can have a downside as well as being advantageous. In the following 2 sections, we review the key properties of retroviral vectors that have made them such attractive gene delivery vehicles and also point out their current limitations and the steps being taken to improve this vector system.

III. ADVANTAGES OF RETROVIRAL VECTORS

A. Fully Defective Vectors

Retroviral vectors represent a truly defective vector system; none of the viral proteins need to be expressed in the target cell for efficient gene transfer to occur and can simply be provided in *trans* in the producer cell. In addition, no *de novo* viral protein synthesis is needed to maintain or repair the integrated provirus. This has implications for long-term gene expression in the transduced cells as even a low-level production of viral proteins will increase the likelihood of an unwanted immune response being triggered against the transduced cells (in addition to any immune response that may be mounted against the transgene itself). Such vector antigenicity is largely responsible for the transient nature of the gene expression seen with current adenoviral vectors, although the problem is being aggressively pursued through the development of helper-dependent or ''gutless'' adenoviral vectors.

B. Vector Integration

A major advantage of retroviral vectors has been that they integrate into the host cell genome. The only other vector system that allows efficient integration is based on adeno-associated virus, reviewed elsewhere in this book. A great deal is known about the process of retroviral integration, which is carried out by the viral integrase protein. Integrase recognizes sequences at the ends of the LTRs of the DNA provirus (the *att* sites, Fig. 2), and inserts the provirus more or less randomly

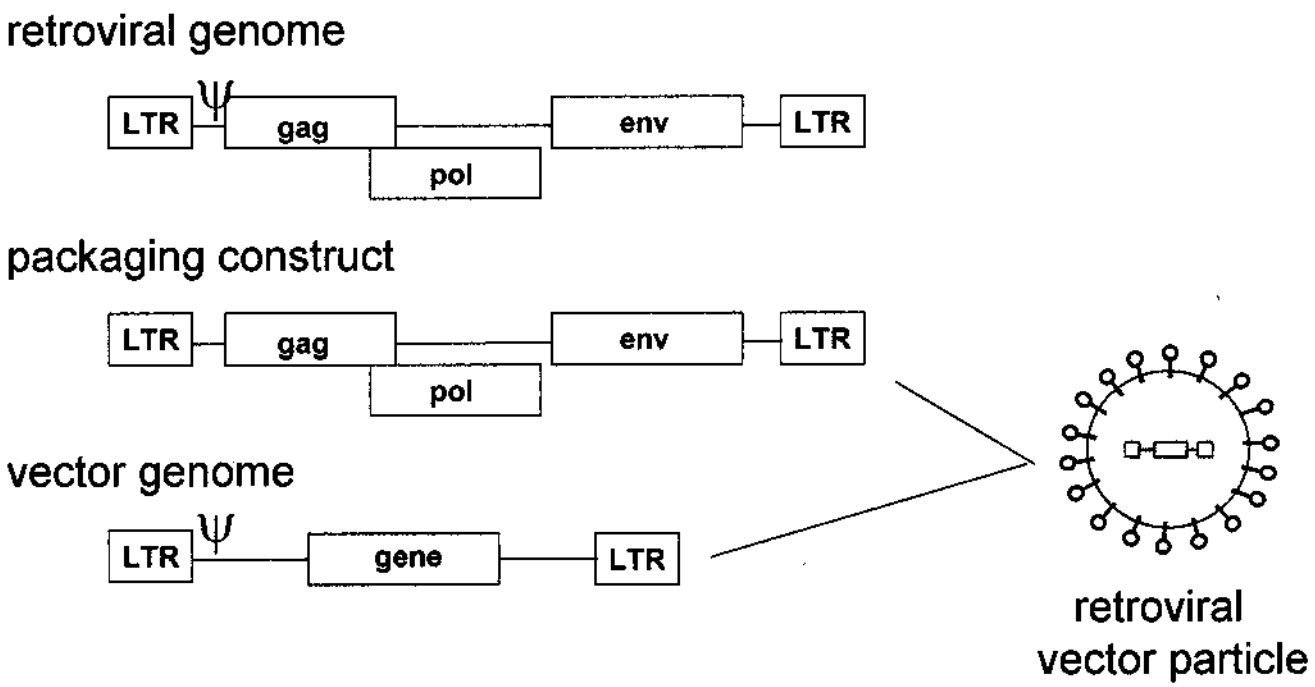

Figure 3 Basic retroviral vector design. The packaging construct provides all the viral proteins in *trans* to the vector genome, which codes for no viral proteins but retains all the necessary *cis* elements. The deletion of the packaging signal from the packaging construct prevents its incorporation into viral particles.

into the host genome, although some sequence preferences have been reported for human immunodeficiency virus-1 (HIV-1) and its vector derivatives (20,21).

The ability of vectors to integrate is a two-edged sword. On the one hand it allows for the possibility of stable long-term gene expression, with the integrated provirus being passed on to all daughter cells. On the other hand, the possibility of insertion into a nonfavorable site also exists, which could influence both the ability of the vector to drive gene expression, and also interfere with the normal functioning of nearby host genes. Most worryingly, the vector insertion event could lead to the activation of an oncogene or inactivation of a tumor suppressor gene, with the risk of subsequent tumor formation. Indeed retroviruses were first identified on the basis of their ability to cause oncogenic transformation, and the possibility of insertional mutagenesis is of great concern. This is discussed in greater detail in Section VII.B.

One possible way around the negative aspects of retroviral integration would be to engineer the integrase protein to direct integration only into certain preselected regions of the host cell genome. The rationale for such an approach is based on the integration site preference exhibited by the related integrase protein of certain yeast transposable elements, such as Ty3, which preferentially integrates upstream of Pol III promoters (22). To date, chimeras between the integrase proteins of Ty3 and MuLV have been shown to be functional, although no redirection of MuLV integration has yet been demonstrated (23).

In a different approach, site-specific integration is being attempted through engineering of the retroviral integrase protein to contain additional DNA targeting domains that will direct the integration complex to specific sites. Some specificity of site selection has been demonstrated in vitro for chimeras containing DNA-binding zinc finger proteins (24), but the ability to redirect integration in vivo has not yet been demonstrated (reviewed in 25).

C. Modularity of Components

The 3 basic components of a retroviral vector—the structural core particle and its enzymes, the envelope-embedded fusion protein, and the vector genome itself—can be viewed as discrete components that in many cases can be ''mixed and matched'' for custom applications. This flexibility enables the exploitation of the natural variations in host range of different viruses and, in some cases, may allow transduction of a cell that is resistant to standard vector combinations. The clearest example is in the use of different envelope proteins to pseudotype the vector particles. For example, when compared with MuLV vectors containing the amphotropic MuLV Env protein, pseudotyping with VSV-G, RD114, 10A1, or gibbon ape leukemia virus (GALV) Env proteins enhances the transduction of primary human hematopoietic cells (26–31).

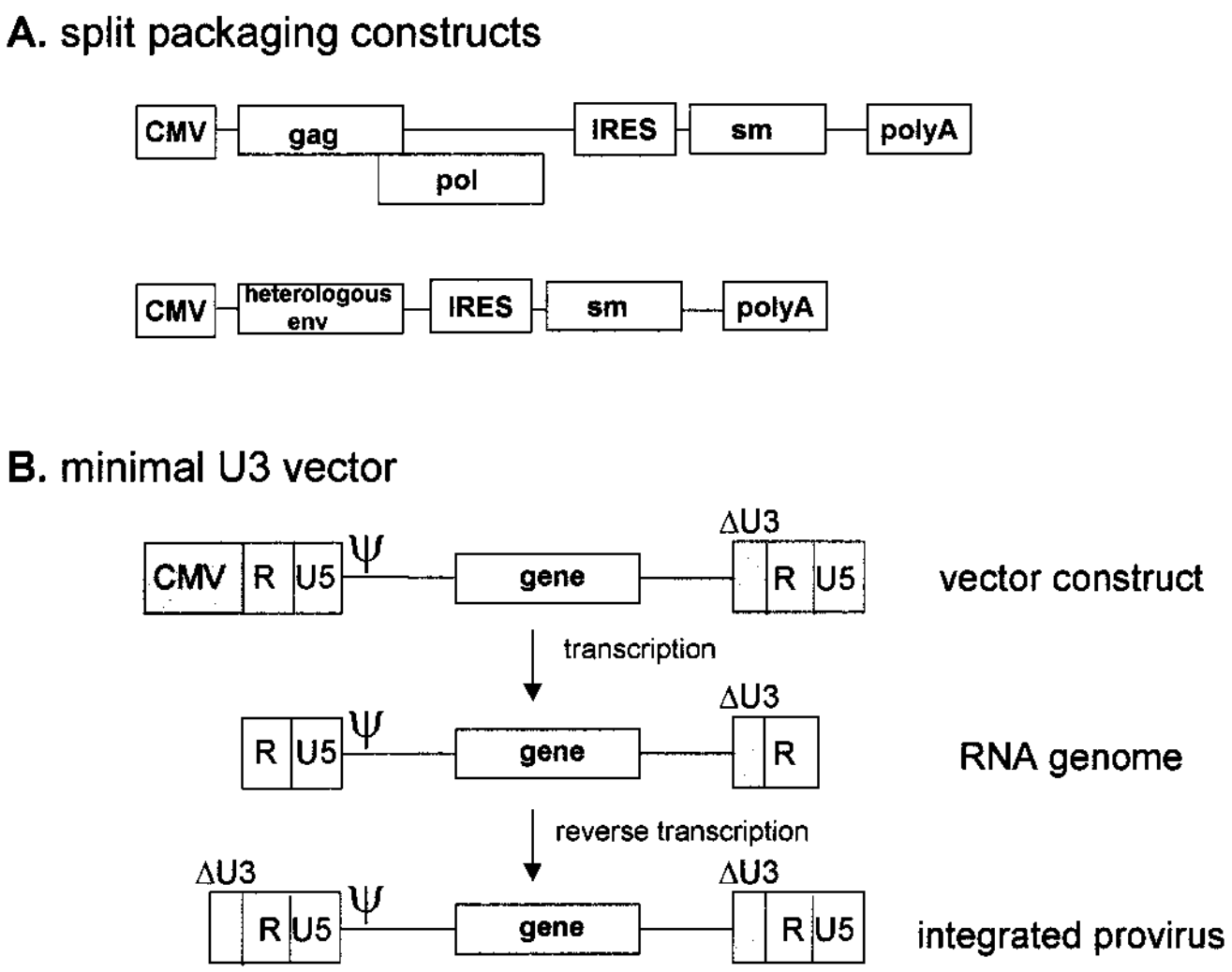

Figure 4 Improvements in vector design. (A) Packaging construct. The Gag-Pol and Env proteins are separated onto 2 different plasmids, and safety is further increased by the use of heterologous envelope proteins. Expression is maximized from the packaging construct through the use of a non-LTR promoter (CMV) and linkage to a selectable marker (sm). (B) Vector construct. The U3 sequences are replaced at the 5′ LTR and minimized in the 3′ LTR. Following reverse transcription, the deleted U3 sequences (ΔU3) are copied into both the 5′ and 3′ LTRs of the provirus. The 5′ LTR therefore has greatly reduced promoter activity. This is the basis of self-inactivating (SIN) vectors.

In addition, different core and vector components can also be used. The combination of GALV cores with MuLV or GALV vector genomes has been reported to enhance the transduction of certain human cell lines (32), and both hybrid retroviral/lentiviral genomes (33) and cores (34) have been constructed that may offer new properties. Finally, the vector LTRs can also be manipulated to improve gene expression in certain cell types. The related myeloproliferative sarcoma virus (MPSV) LTR has been used in place of the MuLV LTR to enhance gene expression in embryonic stem cells (35), and the enhancer elements of the native LTR can be replaced with more specific sequences in order to optimize gene expression in a given host cell (see Section IV.B).

D. Flexibility of Gene Expression Strategies

Because the 5′ retroviral LTR is itself a promoter, the simplest vector design uses the LTR to drive transgene expression (36) (Fig. 5). In addition, the use of heterologous promoters placed internally within the retroviral transcription unit increase the flexibility of gene expression, as constitutive, inducible, or tissue-specific promoters can be included. Expression of more

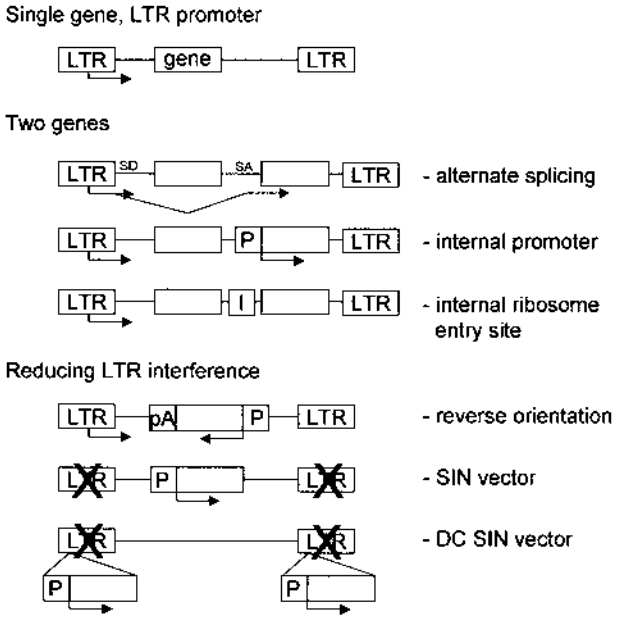

Figure 5 Gene expression from retroviral vectors. Strategies to drive expression of 1 or 2 genes, and to minimize interference by the 5′ LTR promoter. SD, splice donor; SA, splice acceptor; P, promoter; I, internal ribosome entry site; pA, polyA sequences; SIN, self-inactivating; DC, double copy.

than 1 transgene can be achieved through the use of both the LTR and an internal promoter, or by exploiting the differential splicing of the vector that occurs when the major splice acceptor site upstream of the *env* gene is retained (37). In addition, the expression of 2 genes can be linked by the use of an internal ribosome entry site (38).

The transcriptional activity of the LTR can in some cases be a problem, interfering with the activity of internal promoters (promoter interference) (39). These problems can be reduced by the use of SIN vectors, where extensive sequences of the LTR are deleted to effectively silence its promoter. The level of gene expression from integrated vectors can also be increased through the use of double copy vectors (40). Here, the therapeutic gene and its promoter are inserted into the 3′ LTR itself, with the result that after reverse transcription, 2 copies of this expression cassette are created in the provirus.

IV. CURRENT LIMITATIONS OF RETROVIRAL VECTORS

There are several problems investigators face in developing retroviral vectors that will be clinically effective. First, the vector genomes themselves have limited capacity for insertion of foreign sequences, based on the packaging constraints imposed by the viral core proteins; MuLV-based vectors cannot greatly exceed the 8.3-kb size of the MuLV genome. The stability of the engineered vectors can also be a concern. The presence of 2 copies of the vector genome in a viral particle and the process of reverse transcription both contribute to a relatively high level of rearrangement and instability, which is also influenced by the nature of the inserted sequences. The retroviral life cycle and the process of reverse transcription preclude the use of intron-containing sequences in a vector unless the gene is inserted in reverse orientation in the vector, and even with a cDNA copy of a gene, cryptic splice sites can become apparent when the gene is placed inside the retroviral vector.

Major improvements are also required in the overall efficiency of delivery of retroviral vectors. This will involve both the Env-directed entry process and overcoming any postentry blocks to transduction that might occur in specific cells, notably the inability to transduce nondividing cells. Once delivered to a target cell, improvements will be needed in the ability of the vectors to sustain gene expression long term, and for the therapeutic gene and its controlling sequences to respond to appropriate stimuli—be they natural developmental or physiological signals, or regulatory drugs administered to the patient. Finally, cost-effective ways to manufacture the vector at high enough titers will be required, with appropriate assurances of safety.

Specific issues related to the transduction of nondividing cells are dealt with in Section V.B, and manufacturing and safety concerns are discussed in Section VII. Below, we review current attempts to improve the specificity of gene delivery with retroviral vectors and the strategies designed to improve gene expression from those vectors.

A. Obtaining Efficient and Specific Gene Delivery

An ideal retroviral vector is one that could specifically home in on its target cell in the body and limit its transduction to only that type of cell. This would allow the in vivo delivery of the vector and greatly facilitate the clinical procedure for gene therapy. The entry of a retrovirus into a cell is determined in large part by the properties of its envelope glycoprotein and the specificity of the interaction of that protein with its receptor. To some extent, these restrictions can be circumvented by the use of heterologous fusion proteins to pseudotype the vector particles. However, other host range restrictions also exist, including several postentry blocks to transduction. An obvious example of this is the requirement for nuclear membrane breakdown for MuLV entry to the nucleus, but other less well-characterized resistance mechanisms are also present in some cells. For example, certain human cell lines that were poorly transduced by MuLV-based vectors were shown to be more susceptible to transduction by vectors based on GALV (32). In addition, the LTR promoter can also be considered a determinant of tropism and if gene expression is to be driven from that promoter, then its function in a particular cell type will also be an important consideration.

Current clinical protocols for retroviral vectors use an ex vivo approach. Because many of the cells to be transduced

by the vectors express a high level of the natural amphotropic MuLV receptor and are actively dividing at the time of exposure to the vector (either naturally or as a result of culturing conditions), they are relatively easily transduced by MuLV vectors coated with the amphotropic MuLV Env. An important exception at present are primitive hematopoietic stem cells, which are reported to have a low level of the amphotropic receptor and be poorly transducible (41). However, the use of both the GALV and VSV-G fusion proteins has gone some way toward enhancing transduction, as has the use of lentiviral vectors (42,43).

Pseudotyping with natural viral fusion proteins that interact with different cell surface receptors, such as the amphotropic, xenotropic, polytropic, and 10A1 MuLV Env proteins and the GALV and VSV-G proteins, may provide enhanced transduction of a particular cell type ex vivo, but these are all still broad host range proteins that do not provide much specificity. This therefore limits the ability of such vectors to be useful in vivo because introducing the vectors systemically would result in the particles binding to the majority of cells that they encountered and being diluted out before reaching their target cells. The problem can be quantitated. The human body contains approximately 5×10^{13} cells. Using concentrated stocks of retroviral vectors pseuodtyped with VSV-G (which have been reported to give titers of up to 10^9/mL), and infusing 100 mL of such a vector into a patient, would result in the delivery of about 10^{11} active vector particles. Even if every vector particle were 100% efficient, only 1 cell in 500 could possibly be transduced, and this scenario does not take into account the sequestering of the vectors into the first tissue that they come into contact with (typically the lungs), or the inactivation of particles in vivo by both innate immunity and more specific humoral responses. In addition, there will very likely be detrimental side effects resulting from the delivery of the vector to nontarget tissues as a result of the broad host range of the vector. An important step towards the in vivo use of retroviral vectors will clearly be the development of retroviral particles that can preferentially bind to and transduce their target cells and can be manufactured at a high titer.

B. Targeting Retroviral Vectors

Efforts to target retroviral vectors to specific cell types have concentrated largely on engineering natural retroviral envelope proteins, and in particular the rodent cell-specific ecotropic Moloney MuLV protein [reviewed in (44,45)]. Retroviral Env proteins exist as an oligomeric complex (in the case of MuLV Env, probably a trimer), comprising 2 subunits, the surface (SU) protein that contains the receptor recognition domain and the transmembrane (TM) protein that anchors the complex in the retroviral envelope (Fig. 6A). The binding of SU to a specific cell surface receptor is believed to trigger conformational changes in SU and the associated TM protein that result in the exposure of a hydrophobic stretch of amino acids at the N-terminus of TM, the fusion peptide, and the subsequent fusion between viral and host cell membranes (Fig. 6B). The challenge of engineering Env has been to redirect

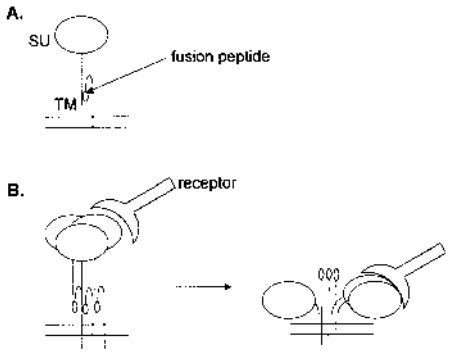

Figure 6 Retroviral Env protein. (A) The Env protein consists of 2 noncovalently linked subunits, the SU protein, which contains the residues that interact with the receptor, and the membrane-anchored TM protein, which promotes the fusion of viral and host cell membranes. At the N-terminus of TM is a stretch of hydrophobic amino acids called the fusion peptide. (B) The retroviral Env protein is oligomeric and MuLV Env probably exists as a trimer. Following binding to its receptor, the Env complex is believed to undergo conformational changes that result in the exposure of the fusion peptide, enabling it to interact with the host cell membrane and initiate the fusion process.

binding of the SU moiety to a heterologous cell surface molecule, while retaining the ability of such an interaction to recapitulate the natural postbinding events that lead to fusion. This has proved a daunting task and, despite early optimism in the field, there have now been numerous reports of failure of targeting strategies with MuLV Env proteins (45–48). Even the few reported success stories appear to be ligand speicifc (49), and a robust and general strategy for targeting MuLV Env proteins has not been forthcoming. The Env protein of spleen necrosis virus may be more amenable to engineering (50,51), although success here also appears to be ligand specific (52)

Two broad approaches have been taken to produce targeted envelope proteins (Fig. 7). First, the natural receptor-binding domain of the SU protein can be replaced with a ligand or single-chain antibody designed to bind to a specific cell surface molecule on the target cell. A whole range of receptors have been targeted in this way, but the difficulty remains that even when specific binding can be obtained between the engineered vector and the target cell receptor, the subsequent fusion event is not triggered and gene transfer is correspondingly low (46,48). It is apparent that engineering the receptor-binding domain of SU to redirect binding while maintaining the ability of the envelope protein to carry out fusion will require a better understanding of the structure–function relationships within the envelope protein complex. The 3-dimensional structure of the receptor-binding domain of the murine ecotropic (Friend strain) SU protein is available (53), and several structure–function studies have delineated various functional domains within MuLV Env that shed light on the pathway of

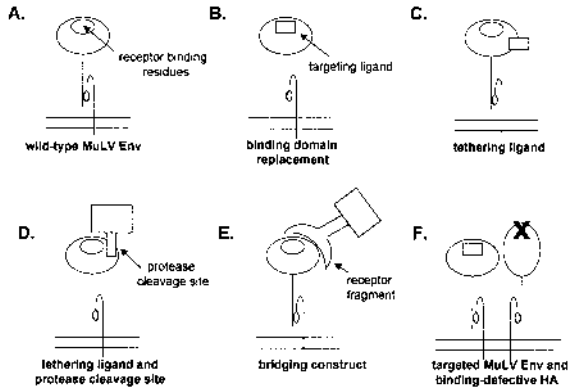

Figure 7 Targeted Env proteins. (A) Wild-type Env protein. (B) Targeting to heterologous receptors: the region of SU that interacts with the natural receptor is replaced by a heterologous binding ligand, such as a small peptide or scFv insert. (C, D) Tethering strategies: the natural receptor-binding interaction is retained, but the vectors are first concentrated on target cells by the action of the additional targeting ligand. In some cases (D), the targeting ligand obscures the natural receptor binding site, which is only revealed after the binding ligand has been removed, for example, by proteolytic cleavage. (E) Bridging strategy: a chimeric protein comprising a soluble receptor and a targeting ligand binds the vector to target cells. The soluble receptor activates the Env protein, allowing fusion to occur with a cell that does not express the natural receptor. (F) Coexpression strategy: receptor binding and fusion functions are separated between 2 different molecules. For example, target cell binding is directed by a chimeric MuLV Env and the fusion component is provided by a binding-defective but fusion-competent influenza HA protein.

signal transmission within the Env protein complex (54–57). Hopefully, such information will allow a more rational approach to engineering retroviral Env proteins in the future.

In a second broad approach to Env targeting that could be called ''tethering,'' the interaction with the native receptor is maintained so that entry occurs through the natural route, but the vectors are concentrated on certain cell types or at certain sites by the presence of an additional binding moiety. The insertion of a collagen-binding ligand into the ecotropic MuLV Env protein, for example, did not perturb the ability of the protein to transduce rodent cells but did allow an effective concentration of the vector at sites of collagen deposition (58). More recently, incorporating this targeting motif into amphotropic (human cell tropic) MuLv Env proteins has resulted in enhanced gene delivery to sites of wound healing following balloon angioplasty, and allowed in vivo gene delivery to human cancer xenografts in nude mice following systemic administration (59).

A different strategy to concentrate vectors on cells expressing the epidermal growth factor (EGF) receptor has been reported that uses a chimeric MuLV Env protein where the natural receptor-binding site is initially blocked by an EGF moiety (60). Following binding to the EGF receptor-expressing cells, the EGF ligand is removed by the action of a protease (envisioned to be present on the cell surface and which can therefore be made cell specific). This cleavage event frees the Env protein to interact with its natural receptor and to subsequently enter the cells. Such a strategy seems particularly suited to cancer cell targeting.

Recently, ''bridging constructs'' have been described for the Env protein from avian leukosis virus (ALV) that combine an EGF targeting domain with the extracellular domain of the ALV receptor (61). These hybrid proteins can be preloaded onto ALV Env pseudotyped vectors and can subsequently ''bridge'' them to target cells expressing the EGF receptor (62). The interaction of the receptor fragment with Env protein triggers the normal fusion process, thereby allowing entry of the vector to the EGF receptor-expressing target cells in the absence of the natural receptor. The success of this system appears to be the result of 2 fortuitous properties of the ALV entry process: (1) the receptor is a single membrane-spanning protein that is amenable to engineering as a soluble domain, and (2) the ALV Env is triggered to induce virus–cell fusion in a 2-stage process, requiring both receptor binding to prime the Env and low pH exposure during endocytosis to complete the process (63).

Finally, in a strategy mimicking the 2-protein approach used by several enveloped viruses, notably the paramyxoviruses, it is possible to combine 2 different moieties on a retroviral vector particle—a targeting protein to bind the vectors to specific cells and a fusion protein that will then promote entry (64). Using a modified influenza hemagglutinin (HA) protein that is no longer capable of binding to its native receptor, the ubiquitously expressed sialic acid (65), we have demonstrated enhanced entry into Flt-3-expressing cells when this HA is coexpressed with a binding-competent but fusion-defective MuLV Env containing the Flt-3 ligand (66).

C. Sustaining and Regulating Gene Expression

Assuming that efficient gene transfer strategies can be developed, the next issue to be addressed is how to maintain an appropriate level of gene expression. With their ability to integrate into host cell chromosomes and therefore be passed on to daughter cells, retroviral vectors have clear advantages as a delivery system when lifelong gene expression is required. However, maintaining gene expression from an integrated provirus has proved problematic in the past. This is perhaps the largest shortcoming of all present vector systems. Because sustaining gene expression in the target cell is not just a problem facing retroviruses, much of the following discussion applies to gene transfer vectors of all types.

Several factors are involved in maintaining the stable expression of genes after their transfer, which can be broadly divided into two categories. The first set concern the activity of the promoter and regulatory sequences themselves. De-

pending on the site of integration of the vector, host mechanisms may suppress (or inappropriately activate) expression from a promoter, and even if gene expression is initially high, it is frequently not sustained long term. There is a tendency for the cell to recognize foreign promoters (particularly viral promoters such as SV40 and CMV) and inactivate them—for example, by methylation—and the retroviral LTR promoter is subject to suppression, particularly in embryonic cells, although strategies have been developed to reduce this problem (35).

The second mechanism that causes loss of gene activity is due to the fact that, even if gene expression remains active, the transduced cell often loses viability. The body can recognize as foreign a therapeutic gene product and can mount an immune response that will eventually eliminate the gene-engineered cells. Even a normal human protein appears abnormal to an immune system that has never been exposed to it.

The problems identified with the use of constitutive viral promoters in vectors have led to recent attempts to use more authentic regulatory sequences to direct gene expression. The use of a gene's own promoter and regulatory sequences may provide more stable long-term gene expression than can be obtained with current viral promoters, but identifying all the necessary components can be difficult. As an extreme case, the regulatory sequences involved in β-globin expression are spread over nearly 100 kb, and because a retroviral vector can only accommodate 6–8 kb, the minimal functional regulatory sequences need to be identified.

Alternatively, certain key elements can be included in the LTR region to provide a measure of cell-specific expression, as has been demonstrated by the insertion of a minimal tyrosinase promoter into the MuLV U3 region that results in melanoma-specific gene expression (67,68). The use of cell-specific gene expression is likely to be an alternative or supplemental approach to targeting at the cell entry stage, because even if the entry of a vector cannot be restricted to the desired target cell population, its subsequent gene expression could.

Finally, the use of authentic genomic elements in retroviral vectors also holds promise for improving gene expression strategies. Chromosome remodeling sequences such as scaffold attachment regions (69), locus control regions (LCR) (70), and insulator sequences (71) may promote stable gene expression whatever the integration site of the vector. In addition, such elements may reduce possible deleterious effects of the vector transcriptional unit on neighboring host genes. We have recently shown that the ADA LCR and promoter can specifically enhance gene expression from retroviral vectors in T lymphocytes (Ball and Anderson, unpublished data).

An additional factor that can influence gene expression is the environment of the host cell being used to express the therapeutic gene. Even when natural regulatory elements are used, they may not function out of context in a different cellular environment. For example, the insulin enhancer/promoter still cannot direct regulated expression of that protein when expressed in fibroblasts. This highlights further the need to develop vectors that are capable of gene transfer to specific cell types.

Although in some gene therapy scenarios, low levels of essentially unregulated expression may be appropriate (e.g., hemophilia, ADA deficiency), for other situations, regulatable gene expression will be desirable. Many of our important genes are not expressed at the same level all the time, but respond to physiological signals within the body. One approach will be to use regulatory sequences that respond to the body's own physiological signals in the vector, so that the therapeutic gene will function in the same way as normal endogenous genes. However, if knowledge of such signals and the corresponding DNA sequences is insufficient to attempt such a strategy, alternate synthetic gene regulation systems could be used where drugs could be administered to control gene expression, such as with the Tet system (72).

D. Integration and the Risk of Insertional Mutagenesis

The potential for a deleterious outcome following retroviral vector insertion has always been acknowledged. Before 2002, the only example of unintentional tumor production in a retroviral gene transfer experiment in large animals occurred when 3 cases of lymphoma were reported among 10 Rhesus monkeys that had received myeloablative irradiation and then been transplanted with hematopoietic stem cells that had been exposed to a large number of RCR, together with the experimental vector. Subsequent analyses revealed that the cancers resulted from integration of an RCR (not the original retroviral vector), were clonal events, and developed only after long periods (6–7 months) of retroviremia (73,74). In addition, analysis of vector integration sites in 1 of the original 2 ADA patients (A.D.), 10 years after she received 11 separate infusions of retroviral vector-transduced peripheral blood lymphocytes, revealed polyclonal integrations sites, even in a subset of T cells that appeared to have expanded in vivo since that time (3).

However, the news that two children treated in a French gene therapy trial for severe combined immunodeficiency (SCID)-XI (75) has developed leukemia is a worrying development. Furthermore, the integration site of the vector in the leukemic cells are near a known oncogene, LMO2. This is a very clear demonstration of the risk inherent in using integrating vectors.

V. DIVIDING AND NONDIVIDING CELLULAR TARGETS

MuLV, and the vectors derived from it, are only able to infect dividing cells. This is because the preintegration nucleoprotein complex is unable to cross an intact nuclear membrane. In contrast, the prototypical lentivirus HIV-1 has been shown capable of nuclear import even when an intact membrane exists, and HIV-1-derived lentiviral vectors are therefore able to transduce nondividing cells (76). This property of HIV vectors

makes them particularly attractive candidates for gene therapy when the target cell is nondividing and stable integration of the transgene is required. However, as outlined below, the restricted tropism of MuLV for dividing cells can also be used to advantage, limiting transduction to dividing cells such as tumor cells.

A. Tropism for Dividing Cells in Cancer Gene Therapy

The selectivity of MuLV for dividing cells has been exploited in a phase III clinical trial that tested the efficacy of a suicide gene therapy approach to treating glioblastoma multiforma, a malignant brain tumor (77). The rationale of such gene therapies is to insert a gene capable of killing cells into the tumor while protecting the normal brain cells. The vector contains the herpes simplex thymidine kinase (TK) gene, which is able to phosphorylate the drug ganciclovir, resulting in a toxic derivative that is incorporated into DNA. The vector is produced in situ in the residual tumor and peritumor areas, following surgical resection of the tumor, by the injection of a mouse producer cell line that generates the retroviral particles. Although both tumor cells and healthy cells in the area of a growing brain tumor could potentially be transduced, only the tumor cells themselves and the vasculature supplying blood to the tumor are considered likely targets as they will be actively dividing. Tumor killing is achieved by giving the drug ganciclovir to the patient; the TK enzyme converts this to a toxic nucleotide that is incorporated into the DNA of the tumor cells, so killing them. However, despite the encouraging preclinical data (78), this trial has now been suspended due to lack of benefit in the gene therapy-treated patients (79).

B. Lentiviral Vectors Transduce Nondividing Cells

There are many situations where one would want to insert a therapeutic gene into normal nondividing cells. In vivo, only certain blood cells and the cells lining the gastrointestinal system are continually in division, so the majority of potential target cells in the human body are nondividing. Lentiviruses such as HIV-1 are able to infect nondividing cells and the demonstration that HIV vectors could also transduce such cells has been an exciting development in retroviral vector technology (76). The mechanism whereby HIV can infect nondividing cells remains somewhat controversial, but there appears to be more than one mechanism involved in making the HIV preintegration complex karyophilic (80–82). Attempts to transfer into murine retroviral vectors the specific signals from the HIV virus that allow transduction of nondividing cells have not been successful (34), underlying the complexity of the process, although the creation of a nuclear localization signal in the matrix protein of SNV has been reported to increase transduction of growth-arrested canine cells (88).

Vectors constructed from lentiviruses, and HIV in particular, raise safety concerns because of the possibility of a pathogenic RCR arising by recombination. Recently constructed HIV-based vectors contain less than 5% of the HIV genome and do not express any viral proteins. Furthermore, non-HIV envelope proteins such as VSV-G are used to pseudotype the vectors. The safety of such systems is constantly being improved, for example, by the development of CMV-driven SIN vectors (89), or minimal packaging constructs with all the nonessential genes removed (90). Much is known about the pathogenicity of HIV and removal of these genes from a vector would, in theory, produce a crippled RCR, even if one did arise. Finally, vector systems based on nonprimate lentiviruses such as feline immunodeficiency virus and equine infectious anemia virus are also being developed (reviewed in Ref. 91).

VI. NEW APPROACHES

Several new gene transfer systems based on retroviral vectors are currently being developed. As reviewed elsewhere in this book, lentiviral vectors, which have the ability to infect nondividing cells, are an exciting new development. In addition, novel approaches to overcome certain deficiencies in retroviral gene delivery are being taken. For example, hybrid vector systems have been created using adenoviral vectors or herpes simplex virus amplicons as carrier systems to deliver the components of a retroviral packaging system. The principle is that the ''first-stage'' vector system will achieve efficient delivery of the retroviral packaging system to target cells (or in close proximity), with these initial cells then acting as a reservoir to produce retroviral vector particles in situ. Subsequent ''second-stage'' transduction by the retroviral vectors will result in both amplification of the first-stage vector titer and stable integration of the transgene. Recently, complete 2-stage vector systems have been incorporated into single molecule vectors, such as herpes-Epstein Barr amplicons and helper-dependent adenoviral vectors (83–85).

Another new development in the use of retroviral vectors is the use of conditionally replicating retroviruses. Such vectors are being developed to carry suicide genes and are envisioned to have utility for cancer gene therapy, where efficient replication and spread of the virus throughout the tumor will be allowed to occur before treatment with the appropriate prodrug. Improved stability of MuLV viruses carrying exogenous genes through judicious choice of insertion site and transgene has recently been demonstrated (86), and virus replication may be further restricted to the tumor cells through replacement of the permissive U3 promoter/enhancer with tumor-specific elements. For example, restricted replication in prostate cancer cell lines has been demonstrated for a green fluorescent protein (GFP)-expressing MuLV with a chimeric LTR containing the androgen-responsive region from the highly prostate-specific probasin promoter (87).

VII. RETROVIRAL VECTORS AS PHARMACEUTICALS

A. Manufacturing Considerations

Although any consideration of how pharmaceutical companies would be able to manufacture gene therapy vectors was an

irrelevant concern in the 1990s, this has now become a real issue. Retroviral vectors are biological agents that can only be made by living cells, and such systems are not easy for carrying out good manufacturing practice and quality assurance/quality control (QA/QC) assays. Furthermore, the large-scale production of retroviral vectors requires the establishment of producer cell lines that maintain a stable arrangement of vector sequences and sufficiently high vector production levels during the procedure. In addition, the subsequent purification of vectors from the supernatant of producer cells is a relatively cumbersome procedure and some loss of titer is inevitable. For vectors pseudotyped with the amphotropic MuLV Env, much of the loss of activity is due to the relative lability of the Env protein (the 2 subunits are noncovalently attached and can dissociate under conditions of sheer stress). In part, this problem could be countered by the use of a single polypeptide fusion protein such as VSV-G, although the inherent cytotoxicity of this protein makes the use of stable producer cell lines difficult (see Section II.C).

B. Safety Considerations

Currently, the 2 most significant concerns for the use of retroviral vectors in humans stem from the possibility of an RCR arising, or insertional mutagenesis leading to tumor formation. RCR formation can occur during the manufacturing process (which can be screened for) (92), or in the patient following vector administration. Such a virus could result from recombination between the vector and packaging components in the producer cells, even with split packaging cell lines (93), or be due to the acquisition of sequences from endogenous retroviruses. Essentially all mammalian cells have their own endogenous retroviruses that could potentially recombine with the vector to produce a new and possibly pathogenic RCR, and many of these endogenous viruses are still unknown. Although any cell line is suspect, the use of primate or human cells as packaging cells raises the greatest safety concerns in this regard. There is no way to predict the pathogenicity of such potential recombinants, and it would be naive to assume that nonnatural combinations of viral components will not be infectious. For example, it has been demonstrated that if the use of an simian immunodeficiency virus (SIV)-based vector pseudotyped with the amphotropic MuLV Env protein gave rise to an RCR, such a chimeric virus could indeed replicate in vivo in monkeys (94).

The subjects of RCR production, safety, and potential tumor induction were extensively analyzed in a report to the National Institutes of Health recombinant DNA advisory committee (NIH RAC) and the Food and Drug Administration (FDA) (95). The report concluded that the QA/QC procedures required by the FDA make it unlikely that any patient could receive sufficient RCR to be pathogenic. However, the manufacturing and testing processes required to ensure this degree of safety are complex and expensive.

As already discussed, a major concern for the use of retroviral vectors is the possibility of insertional mutagenesis. In response to the serious adverse events in the SCID-XI trial in France, clinical trials with retroviral vectors in the US have been put on clinical hold. Until more is known, it is unclear how significant this risk will be, and whether the use of these vectors for life-threatening diseases with no therapeutic alternatives will still be warranted.

C. In Vivo Use

A major goal of present research in retroviral vector development is the production of a gene therapy vector that could be injected directly into the body. Such in vivo use poses additional problems that must be considered. For example, mouse packaging cells produce retroviral vectors that are rapidly destroyed by human complement, markedly reducing their half-life in vivo and the overall efficiency of gene transfer. The major component of this sensitivity arises from the presence of unique sugar groups on the glycoproteins produced in the murine packaging cells (96). However, such an acute problem can be overcome by the use of other, including human, producer cell lines (97). A humoral immune response against the retroviral vector particles themselves and components of the vector supernatant may also occur. Muul et al. (3) reported persistent antibodies in 1 of the original ADA patients against both fetal calf serum and the MuLV core and Env proteins. Antivector antibodies are a concern when repeat administrations are required, although the problem of preexisting immunity that is seen with adenoviral vectors should not occur with current retroviral vector systems.

VIII. CLINICAL TRIALS

A. Strategies for Gene Delivery in Clinical Applications

There are three categories of somatic cell gene therapy (Table 2). The first and most common is ex vivo, where cells are removed from the body, incubated with a vector, and then returned to the body. This procedure is usually done with blood cells because they are the easiest to remove and return. The second category is in situ, where the vector or a producer cell line is placed directly into the tissues to be transduced. Examples of this include direct injection into tumors, or delivery of vectors into the bronchi for cystic fibrosis therapy. The third category is in vivo, where a vector would be injected directly into the bloodstream but subsequent transduction (or expression) would be restricted to a limited cell population. Although no patients have yet been treated, a clinical protocol has been approved for such a therapy for metastatic colon cancer in the liver, using collagen-targeted retroviral vectors expressing a transdominant cyclin G1 protein (98).

B. Summary of Current Clinical Trials

Currently, there are over 600 approved clinical protocols worldwide. Detailed information is available from the RAC on the 545 protocols that were approved in the US as of the end of 2002, and helpful statistical summaries are available on the website of the Journal of Gene Medicine (http://www.wiley.co.uk/genetherapy/clinical/). The original ADA

Table 2 Gene Delivery Strategies

1. Ex vivo
Cells are removed from body and incubated with vector; engineered cells are then returned (e.g., T lymphocytes for anti-HIV therapy)
2. In situ
Vector or producer cells are placed directly into the tissues to be transduced (e.g., TK vectors in brain tumors)
3. In vivo
Vector would be directly injected into the bloodstream and would home in on its target cells (no examples yet)

deficiency gene therapy trial (2) was begun in 1990, giving gene-corrected autologous T lymphocytes to 2 girls suffering from this disease. ADA deficiency is a rare genetic disorder that produces severe immunodeficiency in children. Patient 1 (A.D.) received a total of 11 infusions, the last being in the summer of 1992. Her total T cell level and her level of transduced T cells have remained essentially constant since then, at approximately 20% and at considerably higher levels than patient 2 (C.C.) (<0.01%). Due to differences in cell harvest and transduction efficiencies, patient 1 received greater than 2 logs more vector-transduced cells than patient 2, who also developed persisting antibodies against components of the gene transfer system. These 2 factors likely resulted in the large discrepancy in the frequency of modified T cells seen in the 2 patients. Although patient 1 in particular may have benefited from gene therapy, because both patients continue to receive PEG-ADA treatment in addition to their gene therapy treatments, no final conclusion can be drawn as to the relative roles of PEG-ADA and gene therapy in their excellent clinical course. More recently, Aiuti et al. (99) reported successful results for ADA gene therapy following transduction of hematopoietic stem cells (HSC) without PEG-ADA treatment.

Only 1 large phase III clinical trial using retroviral vectors has been conducted for the treatment of glioblastoma multiforma. This approach was based on the in situ production of amphotropic retroviral vectors expressing TK from a mouse producer cell line, inoculated into the residual tumor and surrounding areas following tumor resection. The phase III trial included a total of more than 40 centers in North America and Europe and was scheduled to enroll a total of 250 patients, but has been abandoned due to lack of therapeutic effect (79).

In 2000, great excitement was generated by the announcement from Alain Fischer's group in France that gene therapy had been used to effectively cure patients undergoing gene therapy for SCID-XI (75). This is an X-linked disorder where the γc cytokine receptor subunit is missing, and is characterized by early blocks in T and NK cell development. The vector used was based on MFG, expressing the γc subunit under the control of the vector LTR. However, great concern has now been raised by the announcement that two patients have developed leukemia. At the present time, gene therapy trials in the U.S. using retroviral vectors are on clinical hold.

IX. CONCLUSIONS AND FUTURE PROSPECTS

To date, retroviral vectors have been the most commonly used gene delivery vehicle in human gene therapy protocols. The simplicity of their design, their broad host range and their ability to integrate into a cell's genome are responsible for their popularity. As with any viral vector system, the potential for RCR formation remains a concern, even with the latest vector designs, but assays are in place to detect such recombinants and guidelines for the production of clinical grade vectors are well established. The risk of insertional mutagenesis is recognized as a downside of their ability to integrate, and highlights the fact that this therapy should be used only in cases where the benefits outweigh the risks.

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Adenovirus Vectors for Gene Therapy

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I. INTRODUCTION

The emergence of recombinant DNA as a tool to study medicine quickly promulgated the concept of cloned genes as therapeutics. As originally conceived, the concept of gene therapy was simply to introduce a wild-type copy of a deficient gene into cells to restore function *in trans* (1–4). Viewed in this way, the technical challenge was to efficiently deliver the gene to the appropriate cell and have it expressed for sufficient time, or readminister as often as needed, for the therapeutic application. Adenovirus (Ad) gene transfer vectors offer one strategy to achieve this. The focus on Ad for gene transfer was based on basic research establishing the biology of Ad, and the knowledge that Ad efficiently delivers the viral genome to the target cells. Importantly, Ad are not oncogenic in humans, the genomes of common Ad are completely defined, the Ad genome can be easily modified, and recombinant Ad can be readily produced in large quantities and highly concentrated without modifying the ability of the virus to infect cells.

In retrospect, the original goal of using Ad as simple delivery systems to permanently complement genetic defects seems naive. Whereas Ad gene transfer vectors can achieve robust expression of the transgene in many target organs, expression of the transgene is limited in time, resulting from a complex combination of innate and adaptive immune host defenses against the virus (5,6). In this context, Ad vectors in their present form are most useful in applications where transient (days to weeks) expression is sufficient to have the desired therapeutic effect. For applications where persistent expression is required to achieve a therapeutic goal, there are still many challenges before and if Ad vectors will be successful. In this chapter, we summarize the biology of Ad, the construction and use of first-generation Ad vectors, the current status of advanced forms of Ad vectors, clinical applications of Ad vectors, and the future prospects of using Ad in gene transfer applications.

II. BIOLOGY OF ADENOVIRUSES

Adenoviruses are a group of double-stranded DNA viruses that infect a variety of vertebrate hosts, including rodents, chickens and primates. Human Ad have been isolated from several sources including the upper respiratory tracts of military recruits with respiratory infections, adenoids, conjunctiva, and the stool of infants with diarrhea (7). As with other viruses, there is an immune response to Ad infection that includes the production of neutralizing antibodies, defined as antibodies which prevent Ad infection in vitro. Neutralizing sera have been used to distinguish 49 different adenovirus serotypes, which are divided into subgroups A through E (8). The presence of antibodies against one serotype generally protects against reinfection by the same serotype. In the context of gene therapy, serotypes 5 and 2 of the subgroup C have been used most because their structure and biology is well described and there are convenient biological reagents available to produce recombinant subgroup C Ad gene transfer vectors in large quantities. Adenoviruses of subgroups C cause various respiratory infections either as outbreaks in confined groups (e.g., military recruits) or children, which are sometimes associated with conjunctival infections (9). The known predilection of subgroup C in the respiratory tract led to the initial uses of Ad gene therapy for the treatment of cystic fibrosis, and thus the focus on serotypes 2 and 5.

A. Structure

Adenovirus consists of an icosahedral protein capsid of approximately 70–100 nm diameter and, within that capsid,

a single copy of a double-stranded DNA molecule of length approximately 36,000 bp [Fig. 1; reviewed in (10)]. In the context of gene therapy, the fiber, penton base, and hexon are the most important capsid proteins. The 20 triangular faces of the viral capsid are built from hexon, the major capsid protein. The 240 hexon capsomeres in the capsid are each trimers comprising 3 copies of the 105-kDa hexon subunit, with each trimer interacting with 6 others in a pseudoequivalent fashion. The 3-dimensional structure of hexon shows that the homotrimer has loops that project from the capsid surface (11). Capsid proteins VI, VIII, and IX are associated with hexon, and their role is to stabilize the capsid structure. The 12 capsid vertices are made up of the penton capsomere, a complex of 5 copies of the penton base, and 3 copies of fiber. Each penton capsomere interacts with 5 hexon capsomeres, 1 from each of the 5 faces that converge at the vertex. The fiber protein projects outward from the penton base. The DNA is wrapped in the histone-like core protein VII, and there is a terminal protein attached to the 5′ end of each DNA strand.

Neutralizing antibodies are directed primarily against epitopes located on the loops of the hexon. This is expected, as the loops project from the surface of the virus where they are accessible to antibodies. When the primary structures of the capsids of different serotypes are compared, related Ad differ most in these loops, suggesting the selective pressures applied by the immune system result in the emergence of mutations in the external hexon loops (12).

The fiber protein is a trimer consisting of 3 domains: the base, shaft, and knob. The N-terminal base domain interacts with the penton base. The shaft includes an extended domain consisting of variable numbers of a 15 amino acid pseudorepeat. The number of repeats, and therefore the length of the shaft, varies between 23 copies for the group A viruses and 6 copies for the subgroup B viruses. The distal C-terminal domain of the fiber protein, referred to as the ''knob,'' interacts with the high-affinity receptor on the surface of the target cell. The high-affinity receptor for adenoviruses except those of subgroup B is referred to as CAR (coxsackie-adenovirus receptor), reflecting the fact that the coxsackie B viruses and most serotypes of Ad share the same receptor (13,14). CAR is a single membrane-spanning protein with two extracellular immunoglobulin-like domains. Apart from acting as a virus receptor, the function of CAR is unknown.

A sequence motif on the penton base is involved in internalization of the virus after high-affinity CAR–fiber interaction. In serotypes 2 and 5 the amino acid motif arginine-glycine-aspartate (RGD) interacts with $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins of the cell surface, and this interaction is essential for efficient internalization (15).

For adenovirus type 5, the most commonly used Ad for gene transfer vectors, the complete 35,935 bp DNA sequence is known. For convenient reference, the genome is divided into 100 equally sized map units. A detailed transcription map at various time points postinfection is used to divide the genome into interspersed early (E) and late (L) regions [Fig. 2; reviewed in (10)]. There is considerable transcriptional overlap among the genes, making manipulation of some areas of the genome difficult. Each of the 5 early genes is comprised of a complex transcription unit with alternative sites for transcription initiation, termination, and

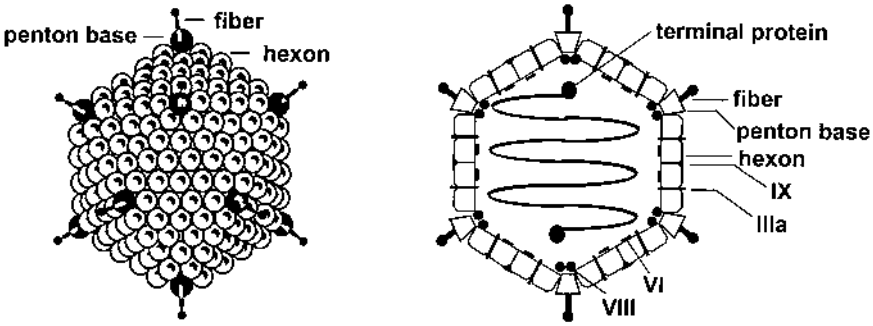

Figure 1 Structure of the adenovirus capsid. Shown (left) is a 3-dimensional representation and (right) a simplified cross-section of the capsid showing the deployment of the capsid proteins and Ad genome. The capsid is an icosahedron with 20 faces and 12 vertices. The faces are composed of hexons, each comprised of trimers of the hexon protein. The hexons are trapezoid shaped, with three loops on top, extending from the face of the capsid. The loops represent the variable regions that differ among serotypes and are the major epitopes for neutralizing antibodies. Proteins IX and VIII are associated with the hexon and are believed to stabilize the capsid. The vertices are composed of a fiber and penton base. The fiber has 3 domains: the base that interacts with penton, the shaft, and the knob. The knob interacts with a high-affinity receptor on the target cell and the shaft holds the virus away from the surface of the cell, depending on the length of the shaft. The penton base interacts with the hexon and the fiber, and contains epitopes that interacts with integrins on the cell surface. The 36-kb double-stranded DNA genome is wrapped around capsid core protein VII and the terminal protein is attached to the two 5′ ends of the Ad genome. (Adapted from Ref. 10.)

splicing. The E1A and E1B genes are transcribed rightward at the left-hand end of the genome close to the DNA replication origin and DNA packaging signal. The E4 region is transcribed leftward at the right-hand end of the genome. Distal to the E1 and E4 regions are the termini of the DNA, which are inverted copies of the same sequence. Replication of the ends of the DNA is achieved by the attachment of terminal protein to the 5′ end of the DNA, which acts as a primer to initiate unidirectional replication. This terminal protein is one of the components of the E2 transcriptional unit, which is transcribed leftward commencing at map unit 75. The remaining early transcription unit is the E3 gene, which is transcribed rightward commencing at map unit 77.

The 5 late genes are expressed after the beginning of DNA replication and encode the viral structural proteins. These late transcripts are all transcribed rightward originating from map unit 17 and contain the same 3-part leader sequence before alternate splicing generates different mature mRNAs.

B. Viral Replication

The Ad viral life cycle is understood best for subgroup C, which is another factor in the choice of Ad5 and 2 as gene therapy vectors (Fig. 3). The knob of the fiber protein binds to the CAR receptor (13), followed by an interaction of the RGD sequence in the penton base with cell surface $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins (15). Excess soluble integrins inhibit Ad internalization but not binding, suggesting that penton base–integrin interaction is instrumental in internalization (16). Ad modified with deletion of the RGD motif replicate effectively in vitro so the penton base–integrin interaction is likely related to efficiency of Ad infection, but is not essential (17). The Ad enters the cell by endocytosis into clathrin-coated pits, a process that can be blocked by dynamin inhibitors (18). After endocytosis, Ad is very rapidly released into the cytoplasm prior to extensive endosome fusion. The virus proceeds rapidly to the nucleus, probably actively transported on microtubules using the dynein motor (19), and then binds to the surface of the nucleus near the nuclear pore (20). Using fluorescent viruses, this process has been shown to be efficient and rapid, with >90% of Ad5 delivered to the nucleus within 1 hr (21). At the nuclear membrane, the DNA and terminal protein are internalized by an unknown mechanism, and are assembled into the nuclear scaffold for active transcription.

With wild-type Ad, the viral E1A gene is transcribed immediately after infection (10). After alternate splicing, the E1 mRNAs are translated into the two E1A proteins

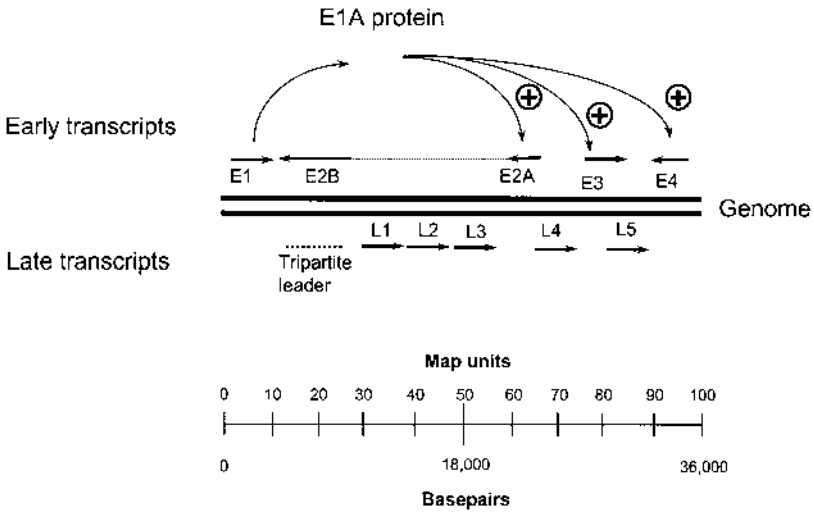

Figure 2 Structure and transcription of the major genes of the adenovirus type 5 genome. Schematic summary of the transcription of adenovirus during lytic infection. The genome is represented as 2 parallel lines and is divided by the scale shown on top into 100 map units (1 map unit = 360 bp). There are 9 major complex transcription units divided into early (above the genome) and late transcripts (below). The 4 early transcripts are produced before the commencement of DNA replication and specify regulatory proteins and proteins required for DNA replication. Upon initial infection of a cell, the E1A protein is produced from transcripts in the E1 region. E1A is a major regulatory factor required for transcription of E1B, E2, E3, and E4. In replication-deficient adenovirus vectors, the E1 region is deleted. Proteins coded by the E2 and E4 regions are required for late gene transcription. The E3 region codes for proteins that help the virus to evade host defenses. All late transcripts rightward originate at the same point and are produced by alternate splicing. The tripartite leader sequence is present at the 5′ end of all late transcripts. The L3 region specifies hexon, the L5 region specifies fiber, and the L2 region specifies penton.

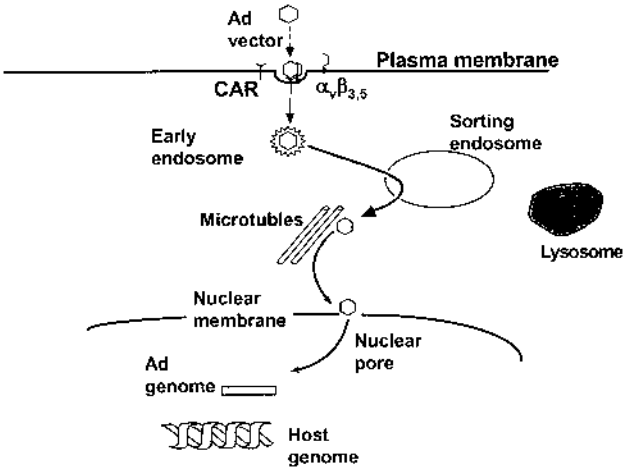

Figure 3 Trafficking of adenovirus from membrane to nucleus. The initial contact between the virus and cell is mediated by the knob of fiber and the coxsackie adenovirus receptor (CAR). This allows the secondary interaction between the penton and $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins, which is required for internalization. The initial internalization is via coated pits, which give rise to coated vesicles. After a very short interval, prior to fusion of early endosomes into sorting endosomes, a conformational change in the viral capsid allows escape of the virus into the cytoplasm. Microtubules carry the virus toward the nucleus. The whole capsid attaches to the outside of the nucleus but only the DNA and terminal protein are inserted into the nucleus itself, where they are assembled onto the nuclear matrix to allow transcription. (Courtesy of P. Leopold, Weill Medical College of Cornell University, New York, NY.)

essential for transcription of other early viral mRNAs. E1A proteins promote the expression of cellular genes needed for DNA replication by interacting with the retinoblastoma susceptibility protein (Rb), which normally suppresses entry into S phase of the cell cycle by complexing with the host transcription factor E2F. E1A also interacts with numerous cellular transcriptional factors to promote the assembly of complexes that promote transcription of other early adenoviral genes. Among the important downstream products induced by E1A is the product of the E1B gene, which blocks the apoptotic pathway through interaction with p53 long enough for a productive viral infection. The E1B 55-kDa protein also complexes with the ORF6 protein from the E4 region to modulate expression of the viral late genes, which begin to be expressed around 6 hr postinfection. At that time, DNA replication begins and the transcription of late genes commences, providing the capsid components that assemble into mature virions (10). The new virions are assembled in the nucleus, necessitating transport of capsid proteins into the nucleus. As the viral infection proceeds, the integrity and viability of the cells decreases, but the mechanism of viral release from the cell is not understood.

In the context of gene therapy, the E3 region is important as it encodes immunosuppressive functions that work through two mechanisms (22). The E3 gp19-kDa protein prevents major histocompatibility complex (MHC) class I-mediated antigen presentation on the cell surface, thereby inhibiting the differentiation of cytotoxic T lymphocytes directed against viral antigens (22). The E3 14.7-kDa and E3 10.4-kDa proteins inhibit apoptosis of infected cells initiated by fas/fas ligand and/or tumor necrosis factor (23). The promoter for the E3 region requires E1 products, and thus in $E1^-$ deleted Ad vectors, the presence or absence of the E3 region is not relevant (see below).

Transcripts from the E2 region specify the three nonhost proteins directly involved in DNA replication: the DNA polymerase, the single-stranded DNA-binding protein (ssDBP) and the preterminal protein (10). Like other viruses, adenovirus has developed a specific strategy for the faithful replication of the ends of its DNA. The last 103 nt at both ends of the genome consist of inverted copies of the identical sequence. The terminal protein binds covalently to the 5′ end and acts as a primer for DNA synthesis by the adenoviral DNA polymerase of the leading strand starting at either end. DNA polymerase proceeds by a strand displacement mechanism, creating a duplex and a displaced strand that is sequestered by the ssDBP and has terminal protein attached to one end. Base pairing of the ends of the single strand creates a panhandle structure with ends identical to those of the duplex. Reformation of duplex from the single-stranded form occurs by the same mechanism with the Ad polymerase initiating at the terminal protein and displacing the ssDBP. Interestingly, the viral genomes undergoing replication are at a different location from those being transcribed (24). DNA is packaged into capsids as directed by a DNA sequence close to the left-hand end of the virus. The efficiency of packaging depends on the length of DNA; genomes greater than 105% or less than 95% of the normal length propagate much less efficiently (25,26).

The E4 region plays important roles in the viral life cycle by promoting the selective expression of viral genes at the expense of cellular genes. For example, the E4-ORF3 and ORF6 proteins inhibit the transport of transcripts of cellular genes from nucleus to cytoplasm while promoting the transport of late viral transcripts (10). The E4 region is therefore essential for viral gene expression and subsequent viral replication.

III. CONSTRUCTION AND USE OF FIRST-GENERATION ADENOVIRUS VECTORS

A. Construction

Although the pathology associated with wild-type adenovirus infections is generally mild, there is a potential risk of using $E1^+$ Ad for gene transfer in that the inflammatory host responses to Ad infection may alter organ function (9). There is also the possibility of overwhelming infection if Ad replication is allowed to progress when there are deficiencies in the

host defense system. Because the E1A products are essential for expression of other early and late genes and for DNA replication, the most direct approach to eliminating replication is to delete the E1A genes. To produce $E1^-$ Ad vectors, the classic approach is to transfect the recombinant $E1^-$ Ad vector genome into the human embryonic kidney cell line 293, a cell line originally established by transforming primary cells with Ad5 (27,28). The 293 cells contain approximately 11 map units of the Ad5 genome, originating at the left-hand end (29).

One example of a so-called ''first-generation'' adenovirus vector expresses the human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA under control of the constitutively highly active cytomegalovirus (CMV) immediate/early promoter (Fig. 4) (30). A polyadenylation site is located following the cDNA, and the whole expression cassette in a left to right orientation replaces the E1A and part of the E1B genes. Because the expression cassette is 5601 bp in length and the E1 deletion is 3062 bp, it is necessary to delete part of the E3 region to construct the vector. Because the E3 region is nonessential in vitro, this deletion does not affect propagation of the replication-deficient virus in 293 cells. However, for some therapeutic genes, if the extra space is not necessary, the E3 region can be retained.

The genomes of first-generation ($E1^-$, $E3^-$) Ad are now typically constructed and amplified as *Escherichia coli* plasmids (Fig. 4), followed by transfection into 293 cells for the production of vector. In one widely used system (31), the Ad genome is created by homologous recombination between a shuttle plasmid and a backbone plasmid. The shuttle plasmid consists of the extreme right- and left-hand ends (map units 1–16 and 97–100) of the Ad genome with the expression cassette for the transgene in the deleted E1 region (map units 2–10 deleted). The backbone plasmid contains most the Ad genome (map units 10–100), except for the extreme left-hand end and a deletion of E3. Recombinants between the shuttle and backbone plasmids containing the whole vector genome are selected by appropriate antibiotic resistance markers.

Once made, a new vector is plaque purified repeatedly in 293 cells (to remove any contaminating wild-type virus) and is then propagated to produce the required amounts of the vector. Under standard laboratory conditions, it is possible to produce up to 2×10^{13} viral particles from fifty 150-mm cell culture plates (about 10^9 293 cells). The recombinant Ad is easily purified from cell lysates on equilibrium cesium chloride density gradients. After purification, the vector is assayed for infectivity by plaquing efficiency on 293 cells, the presence of contaminating replication-competent Ad (RCA) by the plaquing efficiency on A549 cells (an $E1^-$ cell line) (32), and the activity of the transgene (using whatever assay is relevant). Titer on 293 cells gives the titer in plaque-forming units (pfu) per milliliter. This has historically been the activity unit used to standardize doses for experimental animals and patients. However, it has become evident that the pfu is an arbitrary, poorly reproducible measurement, and thus most laboratories now use particle units (pu) as the dosing unit, based on the premise that highly purified viruses made by a standard protocol represent a uniform population of potentially infectious units. The particle count is calculated from the absorbance at 260 nm using the formula $1A_{260} = 1.25 \times 10^{12}$ particles/mL, and is typically 10 to 100 times the titer in pfu (33).

B. In Vitro Studies

The methods outlined above have been used to make a large number of first-generation $E1^-$, $E3^-$ adenoviral vectors. Among the most widely used are those that express readily monitored reporter genes such as β-galactosidase, luciferase, chloramphenicol acetyl transferase, and green fluorescent protein (34,35). As a control, viruses with the same promoter-driving expression of no transgene (AdNull) are used.

Using the reporter gene Ad vectors, there are many studies examining the ease of gene transfer to different primary cells and cell lines. Some primary epithelial cells are easily infected by wild-type adenovirus type 5 and, as expected, are easily transfected by adenoviral vectors. By contrast, macrophages (36) and lymphocytes (37,38) are more difficult to infect and only very high multiplicities of infection in concentrated cell suspensions are effective. The discovery that CAR is the adenoviral receptor partially accounts for the relative ease of infection. There are several studies in which the overexpression of CAR was shown to be sufficient to make an otherwise refractory cell line susceptible to gene transfer by Ad (36,39). But integrins and postinternalization factors must also affect the efficiency of gene transfer.

A large number of cancer cell lines have been shown to be susceptible to gene transfer, including cells derived from hepatoma (40,41), glioblastoma (42), myeloma (43,44), melanoma (45), prostate cancer (46), and ovarian cancer (47,48). In contrast, lymphoma cell lines are resistant to infection (44). Studies in which cells are infected in vitro are instructive in indicating what cell types and therefore diseases might be candidates for adenoviral gene therapy. It is difficult to evaluate if studies with reporter genes show that therapeutic levels of proteins are achievable in any cell type due to the use of Ad vectors with different promoters, reporter genes, multiplicities of infection, and times of exposure.

In cells infected in vitro with E1 deleted, replication-deficient adenovirus vectors, a low level of transcription of early and late genes (49,50), and a small amount of DNA synthesis (51) can be detected. The reason for this is not entirely understood but is hypothesized to result from E1-like activities in the target cell, which support expression of Ad genes. In dividing cells there would also be a high level of E2F, which would support adenoviral transcription. However, measurements of viral load in cultures suggest that this does not translate into the production of infectious viral particles in the absence of contaminating wild-type adenovirus. Although cells may continue to divide after infection, the absolute level of vector does not increase or decrease even if the amount of vector per cell decreases to the point when a small minority of cells are infected. Based on this evidence, the Ad genome is likely to remain episomal and is not integrated into the cellular genome,

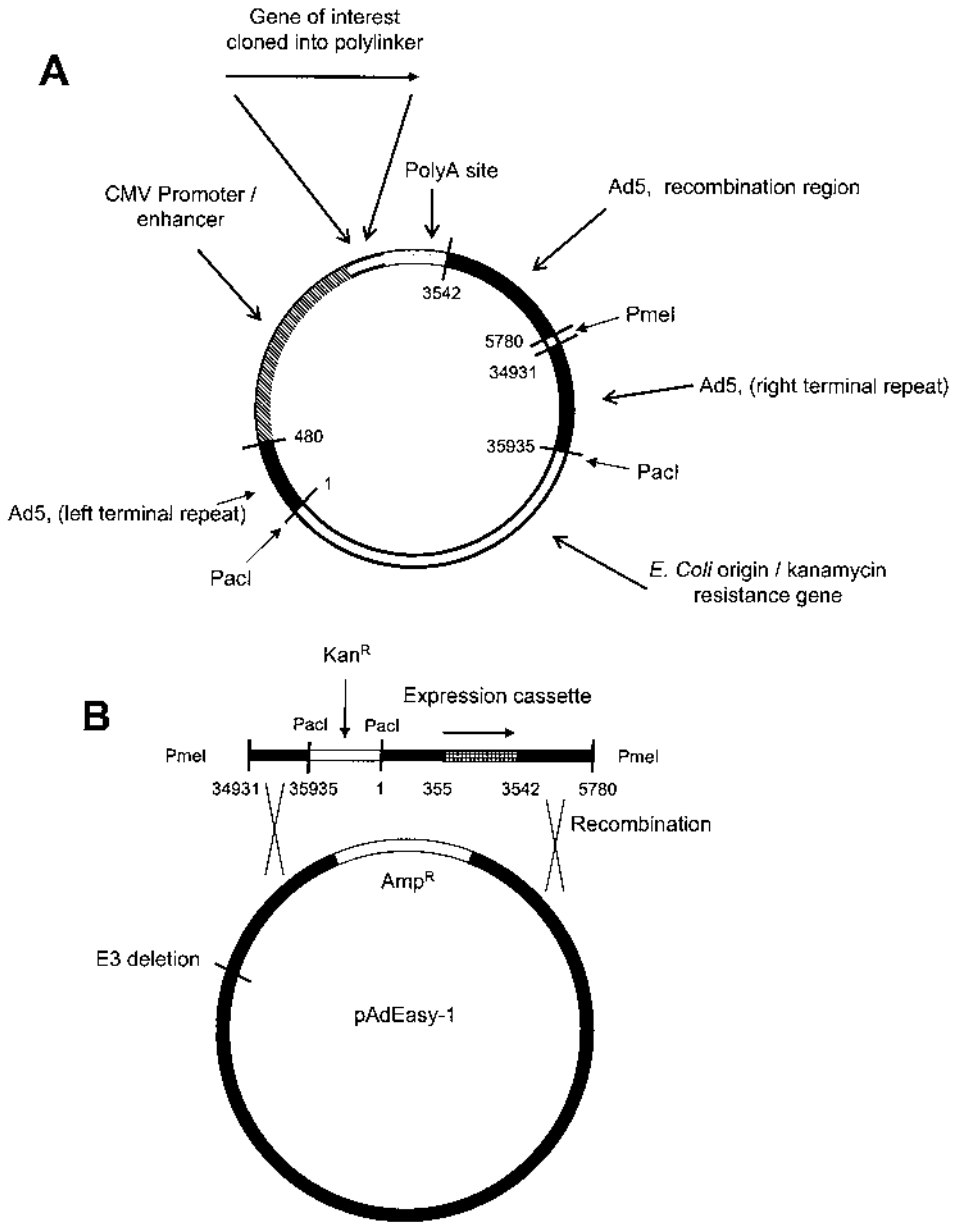

Figure 4 Production of the genome of a replication-deficient adenovirus vector by homologous recombination in *E. coli*. (A) A new cDNA is cloned into the kanamycin-resistant plasmid pShuttleCMV between the CMV promoter and the polyadenylation (poly A) site of SV40. Analogous plasmids are available that allow other promoters to be used (31). The expression cassette lies between 2 portions of the Ad genome, nt 1–480 and 3542–5780. (B) Homologous recombination between PmeI-linearized pShuttleCMV containing the gene of interest and supercoiled pEasy1 results in a kanamycin-resistant plasmid with a full-length adenovirus genome bordered by PacI sites. Plasmid pEasy-1 is used to make E1-E3-E4+ vectors or pEasy-2 to make E1-E3-E4-vectors. The recombinant prepared in large amounts for transfection into E1 complementing cell lines, where it will give rise to the desired vector.

although this is difficult to prove, as it is hard to detect integrated DNA at a very low frequency.

C. In Vivo Studies and Tissue Specificity of Gene Transfer

The feasibility of Ad vector-mediated gene transfer in vitro posed the question of the efficiency of this vector system in vivo. Because Ad gene transfer vectors are made from human adenoviruses, there was no a priori reason to believe they would infect rodents or other model animals. Some early studies used cotton rats because this species had been shown previously to be permissive for replication of human adenoviruses (52–54). For example, intratracheal administration of a replication-deficient virus expressing the reporter gene β-galactosidase to cotton rats resulted in expression of β-galactosidase in the airway epithelium (55). Numerous other animals have been used to demonstrate efficient adenovirus vector-mediated gene transfer, including rats, mice, pigs, rabbits, and nonhuman primates. From these studies, a number of general conclusions can be drawn. Importantly, many tissues can be infected based on the route of administration. As expected from the tropism of Ad5, the transgene delivered to the respiratory epithelium is readily expressed after intranasal or intratracheal administration. But intravenous injection into rodents results primarily in transgene expression in the liver and spleen (56–58). It is not known if the hepatocytes or hepatic endothelium account for this tropism as, surprisingly, the preference for liver does not correspond to the distribution of the CAR receptor among organs (13). Direct injection into the peritoneum (34), kidney (59), pancreas (60), cerebral spinal fluid (61), skeletal muscle (62), brain (63), cardiac muscle (64), coronary artery (65), and many other tissues results in local expression of the transgene. However, the absolute efficiency of gene transfer and expression and leakage to other organs has seldom been calculated, and it is often unclear if therapeutic levels of transgene expression can be achieved.

D. Host Responses

Another important concept that emerged from in vivo studies in experimental animals was the short duration of transgene expression mediated by Ad vectors. Typically, transgene expression levels peak in 1 to 7 days and decline rapidly to undetectable levels by 2 to 4 weeks (Fig. 5). This is true for most routes of administration with the exception of direct injection into a few immunoprivileged tissues such as brain (66–68). Immediately upon administration, the innate immune system serves to eliminate a large amount of vector. Using viral DNA levels as a means to monitor viral clearance from the liver, approximately 90% of an intravenous bolus is cleared after 24 hr (69). Inhibitors of the reticuloendothelium system reduce this early loss of vector, suggesting that macrophages are responsible for early vector clearance (70).

It is known that infection by wild-type human adenoviruses results in a strong immune response in experimental animals and humans. It was not clear whether replication-deficient

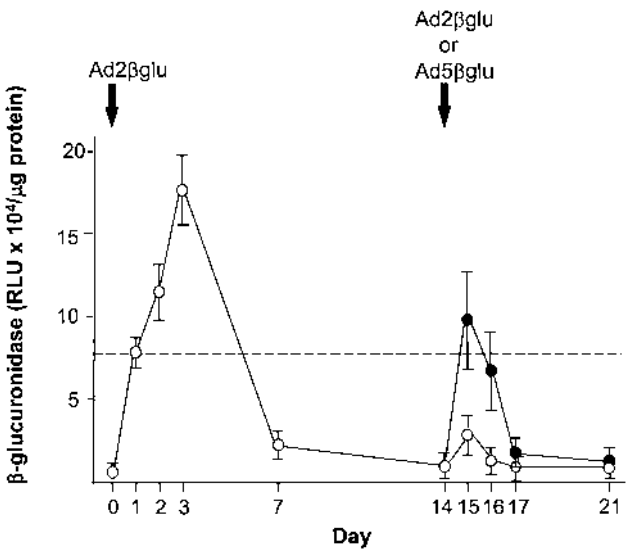

Figure 5 Quantification of β-glucuronidase expression in the lung over time following repeat administration of the same serotype vector or a vector from an alternate serotype. β-Glucuronidase expression in the lung after initial intratracheal administration of Ad2βglu (10^{11} particles), followed 14 days later by intratracheal administration of either the same vector (Ad2βglu, 10^9 pfu, ○) or a vector of the alternate serotype [Ad5βglu, 10^9 pfu, ● (71)].

gene therapy vectors would have the same effect because the net expression of viral genes would be so much lower and the tissues involved would be different from those involved in natural infections. In practice, both cellular and humoral antivector immune responses are observed in rodents after intratracheal (71–73), intravenous (74,75), and intraperitoneal (34) administration. Antibodies against various adenoviral proteins including hexon are induced that can be detected by Western blotting and neutralizing assays. Antivector cytotoxic T lymphocyte (CTL) responses are also observed. The CTL are assumed to eliminate cells infected by the vector in vivo.

When a transgene is used that is foreign to the host, CTL and antibodies are usually, but not always, detected against the expression product of transgene (50,76,77). The basis for this is not fully understood. In classical antigen presentation pathways, a cellular immune response would result from antigens expressed in the antigen-presenting cell and presented in the context of MHC class I. This would imply that Ad infect antigen-presenting cells directly, and there is good evidence both in vivo and in vitro that this occurs (78). But in vivo there is also a strong humoral antitransgene response to many proteins that would not be expected to be taken up by antigen-presenting cells and presented by the class II-dependent pathway. It is possible that apoptosis of Ad-infected cells, followed by uptake of apoptotic cells by antigen-presenting cells, is critical (cross-presentation). The strong antitransgene

product response has proven useful in using Ad as a carrier for genetic vaccination in situations where both cellular and humoral immunity is desired as described below.

These observations lead to the hypothesis that the immune response is essential for the elimination of adenoviral vectors. Although the initial rapid phase of Ad clearance attributed to the innate immune system was equally rapid in immunodeficient mice and immunocompetent mice (79), overall duration of gene expression has been shown to be much longer in immunodeficient mice (79–81). Practically, this suggests ways in which a partial, transient deficiency in the immune system might be exploited to prolong the expression of a therapeutic gene. Conventional immunosuppressants such as corticosteroids (82) and cyclosporin (83–85) have been used to reduce anti-Ad immune responses and increase the duration of transgene expression in experimental animals. The danger of applying this to human is that opportunistic infections or infection from contaminating wild-type adenovirus may result. But greater persistence of Ad vectors can also be achieved via simultaneous systemic administration of molecules such as antibodies against CD40 ligand (86,87) and CTLA4Ig (88,89), which block interaction between T cells and antigen-presenting cells. These immunomodulators could be used locally, possibly coexpressed on the same adenovirus as the therapeutic gene, to provide more specific immunosuppression.

The humoral and cellular responses also evoke immunological memory, which prevents effective gene expression following subsequent administration of the same vector (Fig. 5). Neutralizing antibodies sequester the readministered vector before it infects cells and cause its immune clearance. Thus, the barriers to readministration of a second vector should be serotype-specific, a concept that has been proven in experimental studies (Fig. 5) (71). Cellular immune system memory can also eliminate any readministered vectors that escape neutralization and infect host cells. The determinants of the cellular immune response are more conserved between serotypes and a second vector of different serotype is eliminated faster from immune animals than from naive animals (Fig. 5).

IV. IMPROVED ADENOVIRUS VECTORS

Two salient points emerge from the data discussed above. The first is that only some cells and tissues can be efficiently infected by adenovirus vectors. The second is that there is a strong immune response against adenoviral vectors, which results in elimination of cells infected by the vector and the inability to achieve effective gene transfer and expression following readministration of the same vector. Numerous approaches are being developed that might mitigate these problems.

A. Elimination of Replication Competent Adenovirus

Adenoviral vectors are produced in the 293 cell line, which provides *in trans* the E1 functions that render them conditionally replication competent, permitting vector growth. The difficulty with this approach to propagate vectors is that there is the possibility of homologous recombination between the replication-deficient vector and the chromosomal copy of the Ad5 genome (Fig. 6) (32). This inevitably occurs at a low frequency, resulting in the production of $E1^+$, $E3^-$ replication-competent adenovirus (RCA). Once formed, RCA will outgrow the replication-deficient gene therapy vector in vitro. To minimize the production of RCA, Ad are plaque purified several times on 293 cells and exhaustively tested for the presence of RCA, which is readily detected on the basis of its ability to form plaques on E1 negative cell lines such as the human lung epithelial cell line A549 (32). For clinical studies, adenoviral vectors should be uncontaminated by RCA (level <1 RCA per dose). However, the preparation of Ad vector of this quality is difficult and most in vivo animal and in vitro

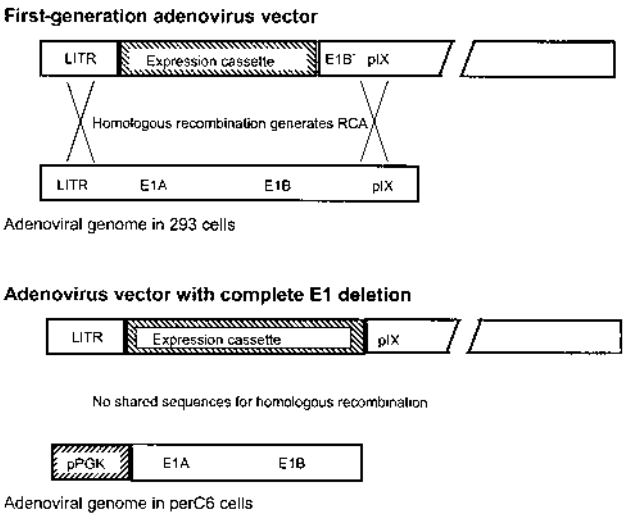

Figure 6 Production of replication-competent adenovirus by homologous recombination between Ad vector and genome of 293 cells. The 293 cell line (open rectangle) contains nucleotides 1 through 4344 from adenovirus type 5, including the left inverted terminal repeat (LITR), the E1A and E1B genes, and the adjacent protein IX gene (pIX). The E1 deletion in most first-generation vectors (top rectangle) stretches from nucleotide 355 through 3328, which is replaced by the expression cassette for the therapeutic gene. Therefore, 2 homologous recombination events (crossed line) can occur that restore the E1 region and give a replication competent (albeit $E3^-$) virus. In the second example, the extent of the E1 deletion in the vector has been extended to encompass all the E1A and E1B genes. At the same time, the E1A and E1B genes in the complementing cell line [i.e., perC6 cells (159)] have no flanking sequence and expression is driven by the phosphoglycerol kinase promoter (p PGK). As a result, there is no homology at either end and only 2 illegitimate recombination events can result in the production of RCA. Therefore, the frequency is very low.

studies are done with preparations with uncharacterized levels of RCA that are probably >1 RCA per 10^8 particles.

To reduce RCA production, two approaches can be used: reduce the size of the trans-complementing E1 region in the cell line or increase the size of the E1 deletion in the vector. Several cell lines have been developed that have less of the Ad genome (compared with 293 cells), while retaining the ability to supply the E1A and E1B functions *in trans* and the high productivity of the 293 cell line (Table 1). The E1 deletion in the first-generation clinical vectors was smaller than optimal, retaining 31% of the 3′ end of the E1B gene. Deleting this sequence in conjunction with cell lines that express E1A/B from nonadenoviral promoter allows production of adenoviruses in circumstances where there is no overlap between the cellular sequence and the vector (Fig. 6B). In these cell lines, RCA is virtually eliminated because it can only arise through two illegitimate recombination events, an occurrence that is very rare.

B. Vectors with Additional Early Gene Deletions

First-generation Ad vectors permit limited Ad gene expression and DNA replication, which probably contributes to the immune response against the vector. In addition, the possibility of making RCA during propagation is a potentially dangerous feature. By making additional mutations or deletions in the Ad genome, both of these problems can be avoided. One implementation is to make an $E1^-$ vector with an E2A mutation that renders the vector replication incompetent at 37°C (90). Such temperature-sensitive vectors can be propagated at 32°C but cannot replicate in the mammalian host at 37°C, even if E1-like activities are present. Further, if homologous recombination in 293 cells during production results in a E1+ viral genome, the ability to replicate in a mammalian host is not restored. In vivo studies show that this defect reduces the inflammatory response following vector administration and

Table 1 Adenovirus Deletions and Complementing Cell Lines

Cell line (genotype)	Deletions complemented	Comments	Ref.
293 ($E1A/B^+$)	$E1^-$	Human embryonic kidney cells transformed by nucleotides 1–4344 of Ad5.	28,29
911 ($E1A/B^+$)	$E1^-$	Human embryonic retinoblast cells containing nucleotides 79–5789 of Ad5. Enhanced plaquing efficiency over 293.	161
perC6 ($E1A/B^+$)	$E1^-$	Nucleotides 459–3510 of Ad5 driven by phosphoglycerate kinase promoter. Reduced production of RCA compared with 911 and 293.	159
293-E4 ($E1A/B^+$, $E4^+$)	$E1^-$, $E4^-$	293 Derivative with E4 gene expression driven by mouse alpha-inhibin promoter.	162
293-ORF6 ($E1A/B^+$, $E4(ORF6)^+$)	$E1^-$, $E4^-$	293 Derivative expressing E4-ORF6 from metallothionein promoter.	93
IGRP2 ($E1A/B^+$, $E4(ORF6/7)^+$)	$E1^-$, $E4^-$	293 Derivative with ORF 6 and 7 of E4 driven by MMTV-LTR promoter.	163
VK2–20 & VK10–9 ($E1A/B^+$, pIX^+ $E4^+$)	$E1^-$, $E4^-$, pIX^-	293 Derivative expressing E4 and pIX from MMTV-LTR or metallothionein promoters allows larger genes to be inserted in E1 region.	164
293 ($E1A/B^+$, pTP^+)	$E1^-$, TP^-	293 Derivative expressing terminal protein inducible by tetracycline.	165
293 ($E1A/B^+$, pTP^+, pol^+)	$E1^-$, TP^-, pol^-	293 Derivative expressing both terminal protein and DNA polymerase.	166
293-C2 ($E1^+$, $E2A^+$)	$E1^-$, $E2A^-$	293 Derivative with 5.9-kb fragment of Ad5 containing E2a region.	167
AE1–2a ($E1A/B^+$, $E2A^+$)	$E1^-$, $E2A^-$	Lung epidermal carcinoma line A549 derivative transformed by E1 and E2A gene under glucocorticoid-responsive promoters.	51
AE1–2a ($E1A/B^+$, $E2A^+$)	$E1^-$, $E2A^-$	293 Derivative with tetracycline repressible E2a gene.	168
293-Cre ($E1A/B^+$, Cre^+)	All viral genes (with lox containing helper)	293 Derivative expressing cre recombinase that excises packaging signal from helper virus.	169

permits longer transgene expression (51,91,92). However, the temperature-sensitive mutation is unstable and partially replication competent at 37°C.

Other mutations in early genes have been used in Ad vectors, including partial and complete E2 and E4 deletions. Both of these genes are essential for viral replication and therefore necessitate the production of cell lines that complement both the E2 or E4 deletion, as well as the E1 deletion. In general, this has been achieved using 293 cells transfected with the appropriate E2 or E4 gene driven by an inducible promoter (Table 1). In a typical example, E4 deleted vectors were constructed in a cell line that expressed the E4 OFR6 behind an inducible metallothionein promoter (93). Cell lines of this type are more difficult to work with than 293 cells, and the efficiency of vector production is often lower. There is inconsistent data on whether additional genomic deletions result in a blunted immune response and whether this translates into longer persistence of transgene expression. Some studies are complicated by the immune response to the foreign transgene and to the vector, and by the tendency of the commonly used CMV promoter to be inactivated over time without vector elimination. For example, one report (94) indicated that a complete E4 deletion has no effect on the time course of gene expression in immunocompetent animals after administration to lung or liver. This is at variance with other reports (50,95,96) showing that E4 deletion results in a reduced immune response and longer transgene expression. The details of vector construction, route of administration, promoter used (97), dose, and genotype of the recipient are critical to the efficacy of E4 (and other) deletion(s). It is likely that studies in humans will be necessary to determine if additional genomic deletions have an impact of the duration of expression of the therapeutic gene and whether this translates into a significant clinical impact.

C. E3 Restored Vectors

The E3 region encodes genes that repress host response to infection through both reducing antigen presentation on the cell surface and protecting infected cells against tumor necrosis factor-α (TNF-α) and/or fas/fasL-mediated apoptosis. E3 is deleted in most adenovirus vectors to make room for the transgene within the length constraints of packaging. Arguing that E3 expression might increase persistence of vectors, several groups have sought to restore one or more E3 function. In mice, expression of the E3gp19K protein has been shown to reduce MHC class I expression in vitro (98) and cytotoxic T lymphocyte levels in vivo (99), but there are contradictory results on whether this translates into prolonged persistence (98,100). However, two studies have shown that the whole E3 region does in fact prolong vector persistence in vivo. This is true whether the E3 region is expressed from an exogenous promoter in the E1 region (101) or expressed from its own promoter in the normal position (102). Because E1 function is required for E3 expression, the latter result is surprising and may indicate that a low level of E3 expression is sufficient to prolong persistence. As with other modifications of the viral backbone, the critical question is if the expression of $E3^+$ vectors would be longer in humans when administered in the route intended for therapy. This has not yet been answered.

D. Helper-dependent Vectors

On the premise that any adenoviral gene expression would cause an immune response, some investigators have developed methods to eliminate all the adenovirus genes from the vector. In fact, the size constraints imposed by some large genes such as dystrophin require that most of the Ad genome be deleted simply to make space for the therapeutic gene (103). An additional benefit of the higher capacity is the possibility of using the endogenous promoter and control elements such as matrix attachment regions to exactly regulate the expression level of the therapeutic gene (104). But deletion of all adenovirus genes requires that those functions be provided *in trans* for vector production. This is achieved by using helper viruses that are deficient in packaging. In an early implementation, a helper virus with defective packaging signals was used that is packaged into virion with much lower efficiency than the therapeutic virus, which has two intact packaging signals. By this method, a mixed lysate is formed with two viruses that differ in size and therefore can be separated on cesium chloride equilibrium density gradients (105).

A refinement of this technology uses the lox/Cre system to negatively select for helper virus in a coinfection of helper and helper-dependent virus vectors (106). In the lox/Cre system, the DNA recombinase Cre from bacteriophage lambda efficiently mediates recombination between lox sites; thus, sequences between two lox sites in the same orientation are deleted. A helper virus called psi5 has been engineered that has lox sites flanking the packaging signal so that Cre recombinase excises the packaging signal and prevents packaging of the genome. The psi5 vector propagates normally in 293 cells but packages inefficiently in 293 derivatives expressing the Cre recombinase. Coinfection of psi5 and helper-dependent vector results primarily in packaging of the helper-dependent vector using proteins specified by the psi5 genome.

Helper-dependent vectors are beginning to attain widespread use and give long-term transgene expression in models where the recipients are immunologically tolerant to the expressed transgene. A helper-dependent vector expressing the human α1-antitrypsin (α1-AT) genomic configuration from the α1-AT promoter can be administered to mice (107) and baboons (108), resulting in prolonged high-level gene expression with little decline over 10 months. Similar data has been reported for factor IX (109), erythropoeitin, and dystrophin. In the case of apoE-deficient mice, the genetic defect that results in high serum cholesterol has been corrected for over 2 years by administration of a helper-dependent vector expressing the wild-type apoE (110). It is clear that the toxicology for helper-dependent vectors is less than for first-generation vectors in some situations, but the acute response to vector, particularly in recipients that are immune to adenovirus type 5, is not affected by use of helper-dependent vectors.

E. Seroswitch Vectors

The induction of neutralizing antibodies is presumably one of the barriers against successful readministration of the same Ad gene transfer vector. In experimental animals, this has been observed for intravenous (111), intratracheal (71,112), and intraperitoneal (34) administration where the vector is exposed to antibodies prior to contact with the tissue. Effective readministration of the same vector has also been demonstrated to be impossible using direct tissue injection (113).

Because prior infection by one Ad does not protect against infection by a different serotype (71), using different serotypes of gene therapy vector should allow readministration of the same transgene. Extensive testing of this concept is difficult because existing vectors are only of serotypes 2 and 5, as the E1 functions of these two viruses can be efficiently complemented by the 293 cell line. For example (Fig. 5), when rats were administered intratracheally with Ad of serotype 2-expressing β-glucuronidase, there was expression that peaked at 3 days and declined to undetectable levels by 14 days. Readministration of the same vector resulted in a very low level of gene expression due to the immune response to the first vector administration. However, a second administration of a vector expressing the same transgene but of serotype 5 resulted in a level of gene expression at day 1 comparable to that seen in a naive rat. The decline to baseline was faster than in naive animals, probably due to the elimination of infected cells by the cellular immune system using epitopes conserved between serotypes 2 and 5 (71).

These data posed the question as to which epitopes are responsible for preventing readministration of the same serotype. Vectors have been constructed with the capsid of serotype 5 but with the fiber gene of serotype 7a. Fortunately, the fiber protein from serotype 7a interacts with the penton base of serotype 5, allowing the assembly of serotype 7a/5 chimeric capsids. In vivo experiments show that the fiber switch from 5 to 7a does not facilitate readministration, suggesting that the immune response to fiber is not the barrier that prevents readministration of the same serotype (114). This is consistent with the concept that the primary humoral immune response is directed to the external loops of the hexon protein. Vectors have been constructed in which the hexon gene of Ad5 has been replaced by that of Ad serotype 2 (115). Even though the level of serological cross-reaction between the pure Ad5 capsid and the variant with hexon from Ad2 was low, the hexon switch did not allow successful readministration in vivo. This illustrates the importance of other arms of the immune system and the diversity of epitopes that are involved in immune response to gene therapy vectors.

It is not clear whether seroswitching is a viable strategy for long-term gene therapy. First, the difficulty of making efficient complementing cell lines for viruses of different subgroups is unknown. Second, the delivery of a therapeutic gene to the target tissue and its subsequent expression would not necessarily be the same among serotypes. Finally, even if this could be achieved, the therapeutic advantage of expressing the transgene once for a few days rather than several times for a few days might not be all that great in terms of genetic disease where persistent gene therapy is needed.

F. Vectors with Modified Tropism

The specificity of adenoviral infections in vitro is dictated by the presence of the CAR receptor and integrins. The role of these receptors in vivo is less certain as the cell types to which a vector is exposed becomes a critical issue. Because a majority of vector administered intravenously in rodents is found in the liver, gene therapy for other tissues requires both detargeting from the normal trafficking route to the liver and a delivery system to the target (116,117). For lung epithelium, intratracheal administration is feasible. Direct injection is possible in some other applications, for example, into the myocardium or directly into a tumor. But vector that is inadvertently injected into capillaries or drains into the circulation through the lymphatic system will find its way to the liver. Alternatively, there are cells types such as lymphocytes (38) that are very difficult to infect with type 5 Ad vectors. These considerations raise questions about whether vectors can be retargeted to cells or tissues of interest, or at least whether the expression of the transgene could be limited to that tissue.

Many vectors use the CMV immediate/early promoter/enhancer, which was chosen on the basis that it directs a high level of transgene expression and is expressed in most tissues studied. However, often expression in a specific tissue or cell type is more desirable and expression in other tissues might be toxic. Therefore, the promoters of genes specific to a cell type have sometimes been used for specific applications (118–120). For example, carcinoembryonic antigen and alpha-fetoprotein (AFP) are tumor-specific antigens that are not expressed by normal cells. When a therapeutic gene is expressed from an Ad vector with an AFP promoter, expression should be confined to specific tumor cells expressing AFP and not normal cells. Kaneko et al. (118) showed this theory to be correct and further demonstrated that the expected selectivity is maintained in vivo. In this context, the vector Av1AFPTK1 [expressing thymidine kinase from herpes simplex virus (HSV-TK) from the AFP promoter] can prevent tumor growth in gancyclovir-treated nude mice implanted with a AFP-expressing tumor cell line but not in identical mice implanted with a control (non-AFP expressing) tumor cell line. In contrast, the vector Av1TK1, which expresses HSV-TK from the Rous sarcoma virus promoter, protects gancyclovir-treated nude mice regardless of which cell line is used to transduce the tumor.

Alternatively, modifications of the fiber/high-affinity receptor interaction or the penton–integrin interaction might be used to modify tissue tropism. In this context, the seroswitch vectors described above (114), as well as capsid chimeras with part of the Ad3 fiber (121,122) or the fiber gene from Ad17 (123), might be more effective in certain tissues because, a priori, different serotypes of wild-type adenoviruses with different known pathologies should target different tissues. In an extensive survey of the tropism of Ad5-derived vectors, but with fibers derived from different serotypes, the fiber gene

of Ad16 was found to be better at targeting fibroblasts and chondrocytes, that of Ad35 better at targeting dendritic cells and melanocytes, and that of Ad50 better at targeting myoblasts and hematopoietic stem cells (124).

Some groups have taken the approach of directly screening for serotypes that replicate preferentially in brain or lung epithelium. In both cases, wild-type strains were screened for efficient replication and certain subgroup D viruses, including serotype 17, were identified to replicate more efficiently. On this basis, a serotype 2 virus with the fiber from Ad17 was constructed and used to study infection of various cell types in vitro. This hybrid is much more efficient at gene transfer to human umbilical vein endothelial cells, neurons, glioma cell lines, and lung epithelial cells than the pure Ad2 gene transfer vector (123). However, it is sufficiently proficient in infecting 293 cells that it can still be propagated and titered for production purposes. It is not known if this translates into better gene transfer efficiency in vivo.

Numerous other approaches to modifying tropism have been identified. For example, the fiber protein can tolerate some manipulation without impairing virus production. The determination of the 3-dimensional structure of fiber in conjunction with mutagenesis studies (125) assists in the identification of amino acids essential for the interaction with CAR and of domains where insertions might be tolerated without grossly affecting structure. An early modification to fiber was the addition of an oligolysine motif to the C-terminal of the fiber protein, giving the virus an affinity for polyanions such as heparin sulfate (126). This profoundly affects the cell types that can be infected in vitro, allowing cells lacking CAR, such as vascular smooth muscle cells and B cells, to be infected. It has also been shown that this oligolysine addition allows for more efficient gene transfer to smooth muscle cells in vivo. Additional manipulations to either the C-terminus or in the HI loop of fiber (summarized in Table 2) have been described to modify the tropism of Ad vectors. The most widely used is the addition of an additional RGD integrin-binding motif to the fiber knob domain, thereby increasing the efficiency of infection of some important cell types, including ovarian cancer cells, fibroblasts, and dendritic cells (127–129). In a study that may point to future developments, phage display technology was used to identify peptide motifs that preferentially target human umbilical vein endothelial cells. When this peptide sequence was incorporated into the HI loop of fiber, adenoviruses were generated with a high preference for endothelium (127,128).

Bispecific antibodies have been used as a reagent to direct Ad toward particular cell types (38,130,131). For example, using a bispecific antibody conjugate with one arm binding the Ad fiber and the other binding the epidermal growth factor (EGF) receptor (130), is was possible to increase the specificity of Ad vectors toward glioma cell lines with low levels of CAR but high levels of the EGF receptor.

The route by which Ad reach the tissue and cell type is complex, especially after intravenous injection. The half-life of Ad in blood is about 2 min, and the interactions with cellular and protein components of blood are unknown (132). In addition the degree to which Ad penetrate endothelium in various tissues is unknown, meaning that in vivo tissue tropism is not a simple outcome of the abundance of CAR primary receptor and integrin secondary receptor. Moreover, in humans there will generally be antibodies against Ad so the formation of complexes with antibody will further complicate tropism. If retargeting is to be successful after an intravenous injection, both detargeting from the normal pathways (117), as well as retargeting to the novel pathways, must be achieved.

Therefore, a number of groups have eliminated the amino acids in the knob of fiber that are essential for interaction with CAR. This makes the resultant vectors difficult to propagate in 293 cells and therefore substitute pseudoreceptor–ligand systems have to be devised. For example, the addition of an HA epitope from the influenza hemagglutinin gene permits a 293 derivative with an anti-HA single-chain antibody to be used for propagation of vectors unable to interact with CAR or integrins (117). The interaction of integrin and penton base has been modified both by elimination of the RGD motif in penton base and by its replacement by the LDV motif, which should promote interaction with $\alpha_4\beta_1$ integrins, characteristic of lymphocytes and monocytes (17).

V. APPLICATIONS

Before commencing clinical studies, batches of vector must be made under the Food and Drug Administration (FDA) current good manufacturing practice and satisfy rigorous lot release criteria. As with all drugs, the identity (now including full DNA sequencing), purity, and potency of the vector must be verified in validated assays. For safety reasons, the absence of RCA is a major concern and vector destined for human usage must contain <1 RCA per dose. As dose increases, the challenge of making vector free of RCA in the 293 cell line becomes more problematic. Vectors must also be verified for the absence of not only human viruses, but also animal viruses, including porcine and bovine viruses that may be carried through from reagents used in cell culture. Taken together, these studies impose a high hurdle of cost and time before a vector suitable for human administration is on hand. Most production is now done in dedicated facilities with high overhead having the trained personnel, facilities, and quality control units.

Human clinical studies also require prior toxicology studies in experimental animals assessing the effects of both vector and transgene expression. There is now considerable human data on the effects of administration of first-generation Ad5-based vectors so the effects of the transgene are usually the focus. Depending on the novelty of the application and the phase to which clinical studies have progressed, toxicology studies may need to conform to FDA good laboratory practice and may require nonhuman primates in addition to rodents. The information derived from toxicology studies is necessarily limited due to biological differences between experimental animals and humans, especially as it relates to the innate and acquired immune response. Experimental animals are naive

Table 2 Examples of Retargeting of Adenovirus Gene Transfer Vectors

Modification	Target	Rationale	Ref.
Ad5 (fiber7a)	Cells expressing Ad7 high-affinity receptors	Replace whole of Ad5 fiber by that of Ad7a (subgroup B).	114
Penton LDV	Lymphocytes, monocytes	Replace RGD $\alpha_v\beta_5$ binding motif in penton base by LDV, which interacts with $\alpha_4\beta1$ integrin.	17
Conjugations to anti-Ad Fabs	Tumor cells	Conjugate ligands (e.g., FGF, folate) to Fab fragments specific to fiber; bind to vector before delivery to cells bearing cognate receptor.	170,171
Bifunctional antibody	Tumor cells	Retarget to EGFR[a] expressing cells; bind virus with bispecific antibody against EGFR and Ad fiber.	130
Bifunctional antibody	Smooth muscle cells, endothelium	Create vector with penton modified to express defined epitope (AdFLAG); use bispecific antibody vs. FLAG and α_v integrin (or E selection) to target cells expressing α_v integrin (or E selectin).	172
Bispecific antibody (anti-CD3)	T cells	Use AdFLAG vector and bispecific antibody (Anti-FLAG, anti-CD3) to target CD3+ T cells.	38
Oligolysine	Cells with surface heparin sulfate	Add lysine residues to C-terminus of fiber.	126,173
Fiber RGD	Cells expressing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins	Add RGD integrin-binding motif to knob of fiber.	39,127
Ad5 (fiber 17)	Endothelium, lung epithelium, brain	Replace fiber gene of Ad2 vector with that of Ad17 (subgroup D).	123
Ad5/9 (short shaft)	Melanoma, glioma, smooth muscle cells	Place knob of Ad9 on shaft of Ad5 after 8 repeats.	174

[a]EGFR, epidermal growth factor receptor.

to human adenoviruses so the immune response to vector is primary, while most humans have preexisting immunity to Ad5 and therefore the immune response is secondary. There are differences in antigen processing and presentation between inbred animals and humans, and the effects of some E3 genes are limited to human MHC proteins. In addition, the potential effects of RCA are different because human adenoviruses do not replicate in rodent cells, but some human Ad are actually oncogenic in rodents but not in humans.

The final step of commencing a clinical study is obtaining regulatory approval, which is also complex. Gene therapy in general and adenovirus in particular are generally perceived as being particularly hazardous and therefore multiple levels of review are required. The local Institutional Review Board (IRB) and Institutional Biosafety Committee (IBC) must approve, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health. The Investigational New Drug application to the FDA represents the last phase of approval. Each group has slightly different concerns, and the final result is a protocol that is conservative yet likely to be safe, ethical and informative. All revisions to the protocol must be approved by all regulatory groups, therefore keeping the protocol current needs well-organized administration. The actual clinical study must be performed under good clinical practice with timely reporting of all adverse events to the FDA, RAC, IRB, and IBC. In addition, many institutions have added a data safety monitoring board to further ensure the safety of gene therapy studies.

Data on models of disease in experimental animals provided sufficient basis to proceed to adenovirus vector-mediated gene transfer in humans. The first human studies were commenced in 1993, and a total of 143 studies are listed in the current update of the database of the RAC. These have generally been small phase I or phase II studies (Table 3). Although the first studies were directed at cystic fibrosis, the currently active studies are focused on cancer and cardiovascular disease. In total, 72% of the listed studies involve cancer with the same vector being studied in a number of different tumors or different patient subsets or concurrently with different concurrent therapies. In general, capsid modified vectors and second-generation vectors have not been used in humans, and it is unknown if the advantages these new vectors sometimes offer in experimental animals apply in humans.

In general, gene transfer of Ad vectors has been safe and well tolerated (133,134). The notable exception is that intravenous administration results in dose-dependent toxicity that has resulted in 1 death (135–138). This reaction is rapid and is believed to be related to the innate immune system. In addition to showing safety, several studies have shown that there is also effective delivery of the vector to the patient and subsequent

Table 3 Clinical Studies with Adenovirus Gene Transfer Vectors[a]

Application	Approach	Indication	Transgene	Route	Investigators (note)
Genetic disease	**Wild-type gene**	Cystic fibrosis	CFTR	Nasal/respiratory tract	– RG Crystal – K McCoy et al. – B Trapnell – RG Crystal (repeat administration)
		Cystic fibrosis	CFTR	Nasal	– M Welch, A Smith (Ad2) – RC Boucher – M Welch (Ad2)
		Cystic fibrosis	CFTR	Respiratory tract aerosol	HL Dorkin (Ad2)
		Cystic fibrosis	CFTR	Intradermal	RG Crystal
		Hemophilia A	FVIII	Intravenous	RA Gruppo et al. (helper dependent)
		OTC deficiency	OTC	Intravenous	M Batshaw (E2a ts)
Cancer	**Growth suppresser gene**	Breast cancer	bclxs	Ex vivo purge, stem cells	MF Clarke
		Breast cancer	mda7	Intratumor	TA Bukholz
		Prostate cancer	p16	Intratumor	JR Gingrich
		Advanced cancer	p53	Intravenous	SG Eckhardt
		Bladder cancer	p53	Intravesicle	LC Pagliaro
		Breast cancer	p53	Intratumor	– M von Mehren – M Christofanilli
		Breast cancer	p53	Ex vivo purge, stem cells	RD Baynes
		Glioma	p53	Intratumor	H Greenberg et al.
		Head/neck squamous cell carcinoma	p53	Intratumor	– GL Clayman – SS Agarwala et al. – CM Bier-Laning – RL Breau et al.
		Hepatic metastasis	p53	Intratumor	CP Belani
		Hepatic metastasis	p53	Intrahepatic artery	RG Amado et al.
		Liver cancer (primary and metastatic)	p53	Hepatic artery	AP Venach
		Lung carcinoma	p53	Lavage	DP Carbone, J Schiller
		NSCLC	p53	Intratumor	– J Roth – T Dobbs – S Swisher – J Schiller
		Oral squamous cell carcinoma	p53	Intratumor	GH Yoo
		Ovarian cancer	p53	Intraperitoneal	– CY Muller – JK Wolf – F Abbas et al.
		Premalignant oral cancer	p53	Intratumor	GL Clayman
		Prostate cancer	p53	Intratumor	– A Beldegrun, RA Figlin – A Pollack
		Bladder cancer	Rb	Intravesicle	P Carroll, EJ Small
	Immunotherapy	Hepatocellular carcinoma	AFP	Intravenous	JS Economou

Table 3 Continued

Application	Approach	Indication	Transgene	Route	Investigators (note)
		Ovarian cancer	Anti-erbB	Intraperitoneal	RV Alvarez, DT Curiel
		Metastatic cancer (melanoma/breast)	B7.1	Intratumor	L Schuchter
		Renal carcinoma	B7.1	Ex vivo, autologous tumor cells	SJ Antonia
		CLL	CD154	Ex vivo, autologous tumor cells	TJ Kipps, W Weirda
		Lung cancer	CD40L	Intratumor	RG Crystal
		CLL	CD40L/IL-2	Ex vivo, autologous tumor cells	M Brenner, S Takahashi
		Acute leukemia	CD40L and IL-2	Ex vivo bone marrow and fibroblasts	M Brenner
		Melanoma	fVII/Fc	Intratumor	A Diesseroth
		Colon cancer	GA733–2	Intradermal	SL Eck
		Melanoma	GM-CSF	Ex vivo, autologous tumor cells	– G Dranoff, R Soiffer – T Suzuki
		NSCLC	GM-CSF	Ex vivo, autologous tumor cells	G Dranoff, R Salgia
		Various	GM-CSF	Ex vivo, autologous tumor cells	T Suzuki
		Ovarian cancer	GM-CSF	Ex vivo, autologous tumor cells	G Dranoff
		AML	GM-CSF	Ex vivo, autologous tumor cells	DJ DeAngelo
		NSCLC	GM-CSF	Ex vivo, autologous tumor cells	JW Smith II
		Glioma	IFN-β	Intratumor	SL Eck
		Melanoma	IFN-γ	Intratumor	JD Rosenblatt
		Liver cancer (Primary or metastatic)	IL-12	Intratumor	MW Sung, S Woo
		Prostate cancer	IL-12	Intratumor	BJ Miles
		Neuorblastoma	IL-2	Ex vivo, autologous tumor cells	– LC Bowman, M Brenner – HV Russell, D Strother
		1. Metastatic/recurrent tumor 2. Sarcoma	TNF-α	Intratumor	N Hann et al.
		NSCLC	IL-7	Ex vivo – autologous DC	SM Dubinett
	Immunotherapy – tumor antigen	Melanoma	MART-1	Various	– JS Economou (iv of id) – S Rosenberg (sc or im)
		Melanoma	MART-1	Subcutaneous	S Rosenberg
		Melanoma	MART-1 & GP100	Ex vivo transduced dendritic cells	F Haluska, JJ Nemunaitis
		Melanoma	MART-1 & GP100	Intradermal	C Cunningham et al.
		Prostate cancer	PSA	Intradermal	DM Lubaroff
	Oncolytic virus	Prostate cancer		Intratumor	– JW Simons – MK Terris – J Cormal
		Prostate cancer		Intravenous	G Wilding
		Prostate cancer		Intratumor	TL DeWeese et al. (Calydon, PSA promoter)

(*Continued*)

Table 3 Continued

Application	Approach	Indication	Transgene	Route	Investigators (note)
		Prostate cancer		Intravenous	– TA Gardner (Osteocalcin promoter) – EJ Small (PSA promoter)
		Osteocytoma		Intravenous	PA Meyers (Osteocalcin promoter)
	Prodrug activation	Prostate cancer	CD/HSV-TK	Intratumor	SO Freytag, JH Kim
		Hepatic metastasis	CD	Intratumor	RG Crystal et al.
		Bladder cancer	HSV-TK	Intravesicle	SP Lerner
		Brain tumor	HSV-TK	Intratumor	– JB Alavi, SL Eck – RG Grossman, S Woo
		Glioblastoma	HSV-TK	Intratumor	I Germano
		Head/neck squamous cell carcinoma	HSV-TK	Intratumor	B O'Malley
		Hepatic metastasis	HSV-TK	Intratumor	MW Sung, S Woo
		Melanoma	HSV-TK	Intratumor	JC Morris
		Mesotheloima	HSV-TK	Intrapleural	SM Albelda
		NSCLC	HSV-TK	Intratumor	WN Rom, S Woo
		Ovarian cancer	HSV-TK	Intraperitoneal	– RV Alvarez, DT Curiel – DG Kieback – DG Kieback (FGFR targeted Ad)
		Prostate cancer	HSV-TK	Intratumor	– SJ Hall, S Woo – D Kadmon – LWK Chung, TA Gardner – E Aguilar-Cordova, EB Butler – PT Scardino et al.
		Retinoblastoma	HSV-TK	Intratumor	RL Hurwitz
	Prodrug activation + marker gene	Ovarian cancer	HSV-TK/somato-statin receptor	Intraperitoneal	MN Barnes III (RGD tropism modified vector)
	Prodrug + oncolytic virus	Prostate cancer	CD/HSV-TK	Intratumor	– JH Kim – SO Freytag, JH Kim
		Penile cancer	CD/HSV-TK	Intratumor	E Aguilar-Cordova et al.
	Prodrug + immuno therapy	Hepatic metastasis	HSV-TK/IL2	Intratumor	MW Sung
Cardiovascular	**Angiogenesis**	CAD	VEGF	Intramyocardium	– RG Crystal (adjunct) – RG Crystal (MIS) – SL Archer et al. – TK Rosengart

Table 3 Continued

Application	Approach	Indication	Transgene	Route	Investigators (note)
					– BM Cohen (catheter)
					– T Sanborn (catheter)
		CAD	FGF-4	Intracoronary	– JS Lee
					– D Churchill et al.
					– V Bethala
		CAD	HIF-1/VP16	Intramyocardial	BL Cmolik et al.
		PVD	PDGF-B	Intradermal/ulcer	– DJ Margolis
					– DL Steed (selective genetics)
		PVD	VEGF	Intramuscular	– RG Crystal et al.
					– BH Annex et al.
		PVD	HIF-1/VP16	Intramuscular	NAF Chronos et al.
	Antirestenosis	AV anastomosis	VEGF-D	Collagen collar in graft	V Fuster
Antiangiogenesis	**Antiangio genesis**	Macular degeneration	PEDF	Intravitreal	PA Campochiaaro
Normals	**Prodrug activation**	None	CD	Intradermal	RG Crystal, BG Harvey
	Prodrug activation	None	CD	Aerosol to airway	RG Crystal, BG Harvey

[a]Compiled from the 143 protocols in the RAC database as of February 2003. Duplicate, closely related protocols from the same investigator are listed only once. Protocols are organized first by general area, therapeutic approach, transgene and then route of administration.
Abbreviations: CFTR, cystic fibrosis transmembrane regulator; OTC, ornithine transcarbamylase; AFP, alpha fetoprotein; CD40D, CD40 ligand; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; PSA, prostate-specific antigen; TNF, tumor necrosis factor; HSV-TK, herpes simplex virus thymidine kinase; CD, cytosine deaminase; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; PDGF, platelet-derived growth factor; PEDF, pigment epithelium-derived factor; NSCLC, non-small-cell lung cancer.

expression of the therapeutic gene. Demonstrations of actual therapeutic benefit will require critical placebo-controlled testing. If an Ad vector does prove to be effective in a large study, there remain substantial production and support issues that will require a significant investment of time and money to provide the reliable supply of a marketed drug.

A. Genetic Diseases

When gene therapy is conceived as the addition of a good copy of a defective gene, it is natural that the initial focus was on genetic diseases such as cystic fibrosis (CF). As described above, the feasibility of intratracheal administration to the lung was demonstrated in animals and the first clinical trial of an adenovirus vector was to the lung of CF patients (139). The initial study was a dose-escalating safety study in which vector was administered to the bronchi in 20 mL of fluid. It became clear that this volume was not well tolerated, so subsequent studies used smaller volumes or a spray of aerosolized vector into the bronchi. The relative accessibility of the site of administration allows that samples of respiratory epithelium can be recovered by bronchial brushing and the presence of vector and therapeutic gene expression can be assessed repetitively. By sensitive quantitative polymerase chain reaction methods, the expression of the CFTR gene delivered by the vector is seen at the site of administration at vector doses of 5×10^8 pfu and greater. The level of vector-derived CFTR mRNA is approximately 5% of the level of expression of the endogenous CFTR gene, and this is believed to be sufficient for therapeutic effect. However, this level of expression is only achieved for a period of a few days and expression rapidly declines to baseline by 30 days (140). Interestingly, the administration of the vector to the airway does not lead to a significant immune response against adenovirus reflected in either neutralizing antibodies (141) or adenovirus-specific T-cell proliferation.

Since the initial safety of vectors expressing CFTR was demonstrated, a study with repetitive administration has been completed. The important result of this study is that expression is reduced or eliminated in subsequent administrations as expected from the data from experimental animals, presumably from the immune response to the first dose (140).

To date, only one study of adenoviral vectors for any metabolic disease has been initiated. This is not surprising because all animal data suggest only a short time of expression of genes

delivered by Ad vectors to most tissues, and the rationale for a human study was not firmly established. Ornithine transcarbamylase (OTC) deficiency is a recessive metabolic disorder of nitrogen metabolism. A $E1^-$, $E4^-$ deleted adenovirus vector expressing the cDNA for OTC was constructed and administered by the intrahepatic route to adults with partial OTC deficiency and safety parameters and the efficiency of gene transfer are currently being assessed (135). During this study it became apparent that a large dose of intravenous vector could be fatal. The use of a helper-dependent vector for the treatment of hemophilia by intravenous administration was stopped when toxicity was observed.

B. Oncology

Due to the unknown safety profile of adenovirus vectors, it is was generally easier to design the early human trials for life-threatening disease. Of the protocols listed by the RAC, 72% involve cancer. Four basic approaches can be identified: local prodrug activation, tumor suppressor genes, immunotherapy, and oncolytic viruses.

One of the first strategies of human gene therapy for cancer was to locally deliver novel enzymes that metabolize prodrugs into the active chemotherapy agent. The general concept of these studies is that local activation of the prodrug in the tumor will concentrate the active agent in the tumor, thus limiting the systemic toxicity from the active drug. Two genes have been used in human clinical trials: HSV-TK and the *E. coli* cytosine deaminase (CD) gene. The HSV-TK protein activates the prodrug ganciclovir to ganciclovir monophosphate, an inhibitor of DNA polymerase. For CD, the prodrug is 5-fluorocytosine, which is activated by CD into the active chemotherapeutic agent 5-fluorouracil. For both agents and activating enzymes, a theoretical benefit is the bystander effect in which active drug would be excreted from the vector-infected cells to kill the neighboring cells of the tumor. Thus, it is not essential to infect every cell of the tumor with the adenovirus vector.

Currently, active protocols apply the prodrug strategy to many types of cancer, including prostate cancer, central nervous system malignancies, ovarian cancer, mesothelioma, hepatic metastases of colon cancer, and squamous cell carcinoma of the head and neck (Table 3). Most studies involve phase I/II studies with intratumoral injection of escalating doses of vector prior to chemotherapy with the prodrug and subsequent scheduled surgery. Tumor removal provides samples for analysis of vector levels, expression of the therapeutic gene, and for analysis of the activation of prodrug and histological studies for cell death and inflammation. The primary end point of these studies is safety, which has been established in some cases.

Tumor suppressor genes have also been used in human clinical studies: p53 (for ovarian cancer, prostate cancer, squamous cancer of the head and neck, breast cancer, non-small-cell lung cancer, hepatic carcinoma, and hepatic metastases); retinoblastoma susceptibility gene (for bladder cancer); mda7 gene for melanoma; and p16 gene for prostate cancer. The concept is that tumor cells have defective tumor suppressor genes that cannot limit cell division, but restoration of the wild-type gene will limit cell division. The theoretical limitation of using antiproliferative genes for tumor therapy is that they will only inhibit proliferation of the cell they infect and have no *cis* effect on neighboring cells. The trial designs are generally similar to those for the prodrug strategy.

A third general approach to adenovirus gene therapy for cancers has been immunostimulatory genes (Table 3). Several different genes have been used in human studies, including CD40 ligand for chronic lymphocytic leukemia, granulocyte macrophage colony-stimulating factor for melanoma and non-small-cell lung cancer, interleukin-2 for neuroblastoma, MART-1 (a melanoma-specific antigen), and B7 (CD80) for melanoma. The concept of immunostimulatory gene therapy is to promote the natural immune surveillance and elimination of tumors that express abnormal antigens by giving a general boost to the cellular immune system (e.g., with IL-2) or a with a tumor-specific antigen (e.g., MART-1) and anti-erbB-2 single-chain antibodies (for ovarian cancer) (Table 3).

A novel antiproliferative approach has been used in human studies using conditionally replication-competent viruses. As described above, the E1B gene is essential to protect adenovirus-infected cells from apoptosis, and its mode of action is through interaction with p53. It follows that E1B function would only be effective in p53 positive cells, but not in p53 deficient tumor cells (i.e., E1B positive viruses would induce apoptosis only in p53 negative tumor cells), whereas normal cells should be protected by the p53 gene. In this context, E1A negative and E1B positive viruses have been demonstrated in animal models to show selective cytolytic effects against tumors. The same viruses have been used in phase I and phase II studies of human ovarian cancers, pancreatic cancer, and head and neck cancer with direct intratumoral injection in conjunction with chemotherapy. These studies are now being extended to phase III testing.

The simplest approach to therapy is to directly administer the vector to the tumor. But for some special applications an ex vivo approach is possible. This is complex due to the need to protect the cells cultured and infected ex vivo from adventitious agents while they are outside the patient. Three approaches have been used. The most common is to infect the tumor cells with an immunostimulatory vector (e.g., Ad expressing a cytokine), expecting to evoke an antitumor immune response upon returning the cells to the patient. Alternatively, dendritic cells, a potent antigen-presenting cell, have been infected ex vivo with Ad expressing a known tumor antigen to evoke an immune response upon returning to the donor. Finally, bone marrow-derived stem cells have been purged of possible contaminants by infection by an anti-proliferative vector prior to returning to a donor.

C. Cardiovascular

With the observation that there is only short-term gene expression from Ad vectors, the question arose as to which medical applications might benefit from transient expression of a therapeutic gene. The general area of tissue repair and engineering

emerged as a good candidate where secreted growth factors would initiate the desired cascade of tissue remodeling, which once initiated would not require the continuous presence of the therapeutic gene. For example, expression of vascular endothelial growth factor (VEGF) after injection of an Ad vector expressing VEGF into rat retroperitoneal fat pad is brief, reverting to baseline after 10 days (Fig. 7). By contrast, the VEGF protein induces an angiogenic response that persists long after the stimulus has disappeared.

On the basis of extensive preclinical testing in animal models of angiogenesis, a number of groups performed clinical studies of adenoviral gene transfer to ischemic heart or ischemic limb in an effort to induce the growth of new vessels and increase blood flow. Although there is no rigorous efficacy data, there is preliminary evidence of improved cardiac function in a number of patients receiving either Ad expressing FGF-4 in the coronary arteries or Ad expressing VEGF121 in the myocardium (142,143). Moreover, when injected into muscles of patients with peripheral vascular disease, Ad expressing VEGF resulted in marginal improvements in some parameters of blood flow to that limb.

D. Normals

The early Ad gene therapy trials demonstrated that, although effective gene transfer could be achieved, persistence of expression is clearly a problem for Ad vectors. There was clearly an immune response to the vector and possibly the transgene itself; however, the biology of that response is not well understood. Animal models, particularly those involving inbred mice, have limited utility in predicting the immune response in humans. To assess the human host response to Ad vectors, 2 trials with normal subjects have been performed using intradermal (144) or intratracheal administration of an Ad vector expressing the *E. coli* CD gene. The intent of these trials is to describe the immune response in humans to an $E1^-$, $E3^-$ Ad vector to provide a background to assess more advanced vectors on a rational basis.

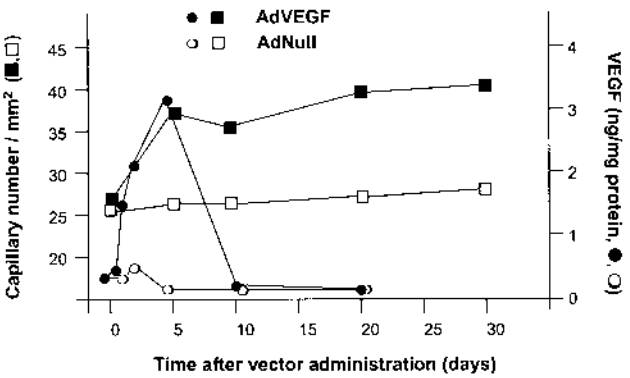

Figure 7 Time course of gene expression and anatomical response after administration of Ad expressing vascular endothelial growth factor (VEGF). The retroperitoneal fat pad of rats was injected with 5×10^8 pfu of either AdVEGF (a first-generation $E1^-E3^-$ vector expressing the 165 amino acid form of human VEGF, solid symbols) or the control vector expressing no transgene (AdNull, open symbols). At intervals, animals were anesthetized, a laporatomy performed, and the fat pad photographed. The number of vessels crossing a circle of 1 cm diameter centered on the injection site were measured (left axis – □, ■). The fat pad was also homogenized and the level of VEGF determined by enzyme-linked immunosorbent assay [right axis – ○, ●; (160)].

VI. FUTURE PROSPECTS

A. Decreasing Vector Elimination

A number of approaches have been developed that should reduce the immune response to Ad vectors, prolong transgene expression, and enhance the efficiency of readministration. The basic hypothesis is that by reducing adenoviral gene expression, there should be a decrease in the host response to the vector and an increase in persistence. This is observed in some experimental animal models but not others. The basic problem posed by these data is whether prolonged persistence and reduced host response will be observed in humans with an administration route compatible with treatment. The only way to answer this question will be to perform the appropriate clinical studies in humans.

The limitations of Ad have prompted some investigators to make hybrids that exploit the wide range of cell types infected by Ad, but allow persistence using features of other viruses such as retroviruses. For example, retroviruses can be produced in situ by coinfection of cells by two Ad vectors (145,146). The first Ad contains the expression cassette for the therapeutic gene flanked by the retrovirus terminal repeats with the necessary *cis* sequences for packaging, all being transcribed from a CMV promoter. The second Ad expresses the the *trans* factors (pol/gag /env) required for assembly of infectious retroviral particles that will then infect the neighboring cells and result in long-term gene therapy. The use of such hybrids has been demonstrated both in vitro and in vivo but does not overcome the need of retroviral vectors for dividing cells, a limitation that might be overcome by making Ad/lentivirus hybrids or Ad/adeno-associated virus hybrids (147,148).

Phage display technology has provided an approach to selecting a peptide sequence with desirable binding properties. This has been exploited in selecting phages that target to different tissues after intravenous injection, presumably through interacting with the endothelium of that tissue. It is likely that these peptide motifs can be incorporated into the knob of the adenovirus fiber to facilitate targeting of adenovirus vectors to a desired tissue. This requires that the knob-modified vector be able to propagate in 293 cells, but strategies have been developed to overcome this production hurdle (117).

B. Applications for Transient Gene Therapy

The technical innovations described above are at best laboratory proofs that will require extensive animal studies before clinical testing. However, the clinical data to date suggests that success with currently available Ad vectors is possible in applications where transient expression might be sufficient. For example, studies of therapeutic angiogenesis for coronary artery disease described above are a prototype of this type of application. Medical indications such as cancer, infectious disease, tissue remodeling (angiogenesis, recovery from surgery, stroke, or injury) are areas where development might be most appropriate. In contrast, metabolic and genetic disease, autoimmune disease, and other chronic conditions would seem to need substantial advances in adenoviral vector design or more likely some kind of hybrid vector before they become treatable on a persistent basis. Importantly, the knowledge of the cellular and host response to Ad infection in humans is still quite rudimentary and will need to be described in much greater detail before more rational approaches to prolonging expression can be devised.

C. Ad Vaccine Carriers

The strong humoral and cellular immune response to the expressed transgene suggests that adenovirus vectors may be effective as vaccines for infectious diseases and cancer. It is possible that there is an inflammatory response to Ad capsids or to residual Ad gene expression that enhances the immune response over that obtained by expression from a plasmid vector. Alternatively, the ability of Ad to directly infect dendritic cells in vivo (78,149) and express the antigen gene may result in antigen presentation by the class I pathway. Examples of exploiting the cellular antitransgene response include eliciting a cellular immune response to tumor antigens as discussed earlier. This property may also be useful in elimination of virus-infected cells (150–152) or malaria (153). It is clear that antigens expressed by Ad also elicit a strong humoral immunity that can be used to block initial infection by viruses (154,155). In the context that most humans have been infected by wild-type adenoviruses and have preexisting antibodies (156), it is possible that use of Ad derived from other mammals may provide more effective vaccines (157,158).

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3

Modified Adenoviruses for Gene Therapy

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I. INTRODUCTION

An intense recent research effort into adenovirus (Ad) biology has revealed crucial steps involved in gene transfer. The tropism of Ads is determined by two distinct virus–cell interactions. Initially, there is high-affinity binding of the fiber knob to the primary receptor, which is the coxsackie-adenovirus receptor (CAR) for most serotypes, including 2 and 5, which are most commonly used for gene therapy approaches (1,2). Binding is followed by interaction between cellular integrins and an arginine-glycine-aspartic acid (RGD) motif located at the penton base (3). The latter event leads to formation of endosomes and viral internalization. Subsequently, the adenoviral DNA is transported to the nucleus and adenoviral protein synthesis, or in the case of replication-deficient Ads, transgene expression, begins. Ad DNA is not frequently integrated into the host genome, thereby resulting in a low risk of mutagenesis. Nevertheless, the limited duration of gene expression may render Ads less desirable for the therapy of metabolic diseases, where long-term expression is needed, but is adequate for cancer gene therapy approaches, where the purpose typically is to kill the target cells. Infection is not dependent on cell-cycle phase. Therefore, both cycling and nondividing cells are infected. Importantly, appealing features of Ad for cancer therapy include stability and unparalleled capacity for gene transfer and expression in vivo. Further, production of high titers of current good manufacturing practices quality Ad, necessary for clinical trials, is well established. Nearly 80% of patients enrolling into gene therapy clinical trials are cancer patients (4). Therefore, Ad is currently the most commonly used clinical agent. Many examples given here will be from the field of cancer gene therapy.

Considering the biology of viral entry, it is logical the degree of gene transfer is determined chiefly by the degree of CAR expression (5–17), reviewed in (18,19). Nevertheless, integrin levels may also play a role. This aspect is not as well understood as various $\alpha_v\beta$-class integrins can mediate the second step of virus entry. $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were the earliest receptors implicated (3,20), but others, such as $\alpha_v\beta_1$ may also be involved, and further molecules may be currently unidentified (21). Also, alternative receptors, such as major histocompatibility complex and heparan sulfate proteoglycan have been implicated although their role is not yet clear (22,23). Finally, some recent data suggests that a heparin sulphate proteoglycan-binding moiety present in the midsection of the fiber may have a role in mediating transduction of hepatocytes (24).

The biodistribution of Ad is not determined only by receptor tropism. In fact, in mice, intravascular Ad results in accumulation mainly in the liver, spleen, heart, lung, and kidneys (25–28), although these tissues may not necessarily be the highest in CAR expression. Instead, the degree of blood flow and the structure of the vasculature in each organ probably contribute to the biodistribution. Importantly, tissue macrophages such as Kupffer cells of the liver have a major role in clearing Ad from blood (29,30). This is an active and non-CAR-mediated process, and uptake via this mechanism leads to rapid degradation of Ad DNA and ineffective transgene expression. Uptake by Kupffer cells saturates when the dose of Ad is increased, resulting in more effective circulation of Ad. This threshold is approximately $1–2 \times 10^{10}$ viral particles in mice, but is not known for humans (28). The threshold effect is probably a major factor contributing to discrepancy between different studies reporting discordant biodistribution and gene transfer rates, as variable dosing has been used. These phenomena also complicate preclinical specificity stud-

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ies, and it is unclear how well murine results correlate with human data.

Although CAR is expressed ubiquitously on most normal epithelial tissues, lack or down-regulation of CAR has been reported for various tumor types, such as ovarian, prostate, lung, breast, and colorectal cancer, as well as melanoma, glioma, and rhabdomyosarcoma (10–13,14,15,17,31–36), reviewed in (18,19). Further, this could be a general phenomenon associated with the carcinogenesis of various tumor types, as inverse correlation with tumor grade has been suggested (14,15). The function of CAR is not well understood but it may be associated with cell adhesion and perhaps cell-cycle regulation. Localization to tight junctions has been suggested (16) as has a role in suppression of tumor growth (15). Interestingly, a preliminary report has suggested inverse correlation between activity of the *RAS-MAPK* pathway and CAR expression (37). For treatment of cancer, these associations are concerning, if confirmed. In particular, the population most desperately needing novel treatments is patients with advanced disease, and if CAR is variably expressed in these tumors, CAR-dependant approaches may not be useful. Thus, targeting of Ad to tumor cells may useful for increasing the clinical efficacy and safety of approaches. Further, considering the widespread expression of CAR, targeting approaches may be advantageous for increasing the specificity of any clinical Ad gene therapy application.

Two main approaches have been used for modification of Ad for the purposes of increased safety and efficacy. Transcriptional targeting restricts expression of transgenes to target tissues, whereas transductional targeting approaches limit the entry of agents to target cells. The former can be achieved by using tissue-specific promoters (TSPs). These promoters are typically associated with genes highly expressed in tumor tissue with lower expression in normal tissues, of which the liver is the most important. Transductional targeting can be achieved by using adapter molecules, genetic modification of the fiber, serotype chimerism, or completely replacing the fiber with heterologous targeting moieties. Also, attempts have been made to use other regions of the capsid for targeting, but little data exists on the feasibility of the approach in vivo. These approaches are discussed in the following section. In addition to introducing new tropism, many applications would benefit from ablation of binding to CAR (38). For systemic treatment, blocking of Kupffer cell uptake may be even more important (30).

II. ADAPTER-BASED TARGETING

The formation of a molecular bridge between Ad and a cell surface receptor constitutes the adapter-based concept of Ad targeting (Fig. 1). Adapter function is performed by bispecific molecules or molecular conjugates that link the Ad to alternative cell surface receptors, often concurrently ablating native CAR tropism. This approach is predicated by the aforementioned two-step entry mechanism of Ad, wherein attachment is distinct from internalization. In this way, alternative means

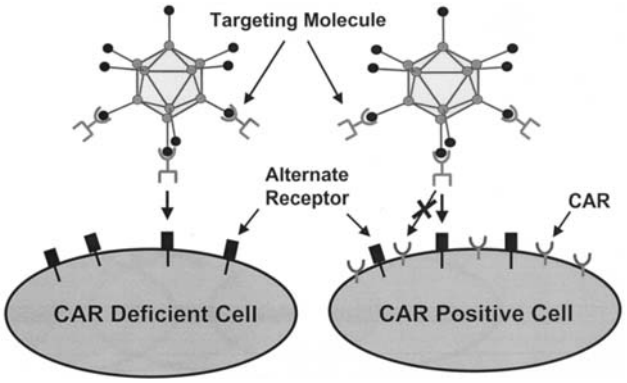

Figure 1 Adapter molecules for epitope-specific adenovirus targeting. The adapter ablates native CAR-based tropism and targets adenovirus to a disease-associated cellular receptor or other surface molecule.

of cellular attachment do not impede Ad cell entry. Bispecific adapter molecules include bispecific antibodies, chemical conjugates between knob-binding single-chain antibodies (Fab) and cell-selective ligands such as folate, Fab-antibody conjugates using antibodies against target cell receptors [e.g., epidermal growth factor receptor (EGFR)], Fab-peptide ligand conjugates [e.g., basic fibroblast growth factor 2 (FGF2)], and recombinant fusion proteins that incorporate Fabs and peptide ligands.

The first in vitro demonstration of Ad targeting via the adapter modality resulted in CAR-independent, folate receptor-mediated cellular uptake of the virion by cancer cells overexpressing this receptor (39). This was accomplished using a bispecific conjugate consisting of a neutralizing Fab chemically linked to folate. A similar targeting adapter consisting of the same Fab fused to FGF2 was used to target Ad vectors to FGF receptor-positive Kaposi's sarcoma and ovarian cancer substrates (40–42). Upon intraperitoneal injection of Ad-Fab-FGF2 coding for herpes simplex virus type I thymidine kinase (HSV-TK) into tumor-bearing mice, survival was prolonged (43). Importantly, decreased hepatic toxicity was demonstrated (44,45). Other Fab–ligand conjugates have been targeted against epithelial cell adhesion molecule, tumor-associated glycoprotein 72, EGFR, CD-40, and others (32,46–52).

An alternative to the chemical conjugate approach was developed by creating a single recombinant fusion molecule formed by the soluble extracellular form of CAR (sCAR) fused to epidermal growth factor (EGF) (53). Increased reporter gene expression was achieved in several EGFR-overexpressing cancer cell lines compared with untargeted Ad or EGFR-negative cells in vitro. EGF-directed targeting to EGFR-positive cells was shown to be dependent on cell surface EGFR density. Initial proof-of-concept studies were followed up with construction of adenoviruses coding for the adapter and capable of achieving production and secretion of sCAR-EGF in human cells. Further, the effect of such retarget-

ing on the oncolytic potency of replication competent agents was tested in vitro and in vivo (54). To improve Ad–sCAR–ligand complex stability, a trimeric sCAR-fibitin-anti-erbB2 single-chain antibody (sFv) adapter molecule was developed (55). The adapter displayed increased affinity to the Ad fiber knob while augmenting gene transfer up to 17-fold in breast and ovarian cancer cell lines.

The above proof-of-principle studies and others have rationalized testing targeting adapters in vivo. A novel bispecific adapter composed of an antiknob Fab chemically conjugated to a monoclonal antibody (9B9) was constructed. 9B9 binds angiotensin-converting enzyme, a surface molecule expressed preferentially on pulmonary capillary endothelium and upregulated in various disease states of the lung (48,56). Following peripheral intravenous injection of the Ad/Fab–9B9 complex, reporter transgene expression and viral DNA in the lung was increased 20-fold over untargeted Ad. Importantly, reporter gene expression in the liver was reduced by 83%.

Adapter-based Ad targeting studies provide compelling evidence that Ad tropism modification can be achieved by targeting alternate cellular receptors, and that this modality augments gene delivery to CAR-negative target cells. Although two-component targeting has shown promising results for retargeting adenovirus to new receptors, such delivery systems have more complex pharmacodynamics and -kinetics, and their stability in humans has not yet been studied. Therefore, one-component systems may be more easily applicable to human trials.

III. GENETIC MODIFICATION OF THE FIBER KNOB

Genetic manipulation may be advantageous over adapters in that all virions harbor an identical modification and lack of stability is less likely. Although conceptually elegant, genetic targeting efforts must work within narrow structural constraints. Key to this approach is the successful modulation of complex protein structure instead of function relationships that result in Ad tropism modification without disrupting native molecular interactions indispensable for proper function. Specifically, genetic modification can affect capsid protein production, fiber trimerization, and packaging of virions. Based on an increasing understanding of native Ad entry biology, development of genetically targeted vectors has rationally focused on the fiber knob domain, the primary determinant of Ad tropism (Fig. 2).

To circumvent variable expression of CAR, targeting ligands have been incorporated into the Ad knob domain without ablating native CAR binding. This results in Ad vectors with expanded, rather than restricted, cell recognition. These efforts are based on rigorous structural analysis of the knob domain and have exploited two separate locations within the knob that tolerate genetic manipulation without loss of fiber function, the C-terminus and the HI loop. Because the C-terminus of the Ad knob is exposed, extension of the knob peptide to include a targeting peptide moiety is conceptually simple.

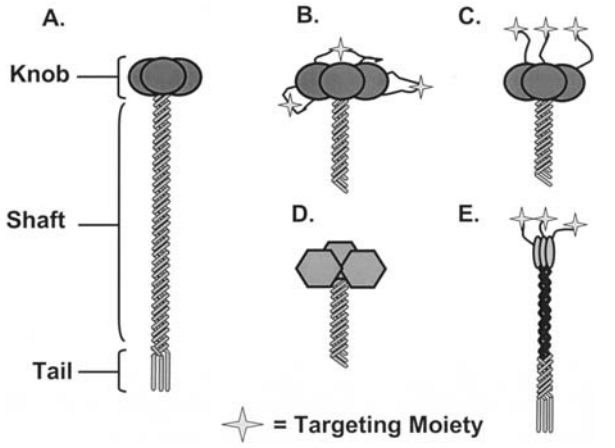

Figure 2 Genetic targeting of adenovirus. (A) Trimeric wild-type adenovirus fiber; tail, shaft, and knob regions are shown. (B) Knob containing targeting ligand in the flexible HI loop. (C) Knob containing a C-terminal targeting ligand. (D) Pseudotyped chimeric fiber bearing an alternate human serotype knob domain. (E) Knobless fiber bearing a heterologous trimerization sequence and a C-terminal targeting moiety.

Ads with C-terminal RGD and polylysine ligands have yielded some promising results in vitro and in vivo, but other peptide ligands were rendered ineffective in the C-terminus context (57). To circumvent this problem, Dmitriev and coworkers introduced an RGD peptide sequence (RGD-4C) into an exposed loop structure that connects beta-sheets H and I (HI-loop) within the knob (31). The expanded tropism of this vector has been useful for several purposes, including carcinomas of the ovary, pancreas, colon, and head and neck, all of which exhibit frequent CAR deficiency (33,35,58–60). Incorporation of various other ligands into the HI-loop has been attempted, but based on the limited number of publications available, the loop may not be a suitable locale for all targeting moieties. The combination of C-terminus and HI-loop targeting has also been performed and was reported to increase gene transfer over either approach alone (61).

A. Serotype Chimerism

Ad fiber pseudotyping refers to genetic replacement of either the entire fiber or just the knob domain with its structural counterpart from another Ad serotype. An Ad5 agent that expressed a chimeric fiber displaying the Ad serotype 3 knob domain (Ad5/3) demonstrated the same CAR-independent cell recognition as Ad3 (62). The use of another Ad5/3 chimeric vector selectively targeted low-CAR lymphoid cell lines in vitro, whereas these cells were refractory to Ad5 infection (63). Ad5/3 has been useful for retargeting Ad5 to low-CAR primary ovarian carcinoma cells and cell lines in vitro and in vivo (27,64).

Importantly, the serotype 3 receptor does not seem to be subject to down-regulation during carcinogenesis, in contrast to CAR. Further, Ad5/3 biodistribution, liver toxicity, and rate of blood clearance in mice appear to not be significantly different from Ad5 when administered intravenously or intraperitoneally, suggesting a good safety profile for Ad5/3. In the context of ovarian cancer, comparison of 5/3 chimerism to infectivity enhancement with RGD-4C was performed and chimerism was found superior. Therefore, it is currently the best available targeting approach for that disease. As with the RGD-4C modification, 5/3 modified viruses are also able to partially escape anti-Ad5 neutralizing antibodies (27). Other chimerism strategies have also been tested (17,65).

B. Knobless Viruses and Other Capsid Proteins for Targeting

Structural conflicts emerging from knob modifications and the observation that fiber-deleted Ad vectors could be produced (66,67) provided the conceptual basis for replacing the native fiber with a chimeric knobless fiber, which would result in ablation of CAR binding. Simultaneous addition of a targeting ligand to the knobless fiber would result in a more specifically targeted Ad. The technical barrier to this approach is the innate trimerization function of the knob, required for proper fiber function and capsid assembly. A solution was devised by using the T4 bacteriophage fibritin protein for trimerization, while proof-of-concept targeting was achieved with a six-histidine (6-His) moiety and cells expressing an artificial 6-His receptor (68). A similar strategy was employed whereby the staphylococcal protein A allowed trimerization and subsequent utilization of an integrin-binding RGD motif or proof-of-concept sFv-mediated targeting (69,70). These approaches could be extremely valuable if they become fully applicable to versatile targeting moeties, such as sFvs, in vivo.

An exciting new approach to Ad targeting is incorporation of targeting proteins into other capsid proteins such as pIII and pIX, which have an accessory function in capsid assembly but have access to the surface of the virion and may thus be usable for incorporation of targeting moieties (71). Also, given that adapter molecules can change the biodistribution of Ad, perhaps alteration of length of the fiber could achieve the same. This hypothesis was tested with promising results, although packaging was compromised with longer shaft extensions (72,73).

IV. TRANSCRIPTIONAL TARGETING WITH TSPs

Transductional targeting approaches attempt to restrict vector entry into target cells. In contrast, transcriptional targeting does not change the tropism of viruses but instead restricts gene expression to target cells. One of the earliest TSPs explored for cancer was the carcinoembryonic antigen promoter, expressed in most gastric, pancreatic, and lung cancers (74). For hepatomas, the promoter of the alpha-fetoprotein has been investigated (75,76), while the L-plastin promoter (LP-P) was used in ovarian and breast cancer cell lines (77,78). Other promoters tested for ovarian cancer include DF3, Cox-2, and midkine (79,80). The Cox-2 promoter has also been explored in the context of gastric carcinomas (81).

Osteocalcin (OC) is a bone protein expressed in osteotropic tumors and differentiated osteoblasts, as well as numerous solid tumors, including osteosarcoma and prostate cancer (82,83). An Ad using the OC promoter to drive *HSV-TK* expression in prostate cancer cells resulted in destruction of tumor cells in vitro and in subcutaneous or bone tumor xenografts. Interestingly, tissue-specific toxicity was seen to bone metastases. OC is expressed in osteoblastic lesions, thus offering the possibility for cotargeting of regenerating bone and tumor. Further, many types of cancers metastatic to the bone could be amenable to treatment.

The secretory leukoprotease inhibitor (SLPI) gene is expressed in several different carcinomas, including ovarian cancer. Its expression in normal organs, such as the liver, is low. Therefore, the SLPI promoter was used to drive transgene expression in ovarian cancer cell lines and primary tumor cells isolated from patient samples (84). The promoter was activated in both cell lines and primary tumor cells in an Ad context in vitro. A murine orthotopic model of peritoneally disseminated ovarian cancer was used to demonstrate high tumor gene expression vs. low liver expression with the SLPI promoter, and that Ad-delivered *HSV-TK* under the control of the SLPI promoter was able to increase survival.

A. Vasculature-specific Promoters

Targeting the endothelium of tumors may be amenable to gene therapy. This tissue may be independent of tumor type and is easily accessible to intravascular vector administration. Also, endothelial cells (ECs) are not malignant, and thus are less sensitive to selection pressure and rarely gain resistance to treatment. E-selectin expression is minimal in normal blood vessels but high in the capillaries of tumors, and the promoter was used for driving gene expression in an Ad. Upon infection, EC cell lines expressed high levels of reporter gene expression, whereas non-EC cell lines showed low expression. The addition of TNF-α, an inducer of the promoter, further increased the E-selectin's activity (85). The murine preproendothelin-1 (PPE-1) promoter was also used as a TSP for adenoviral-mediated delivery to EC cells. Systemic administration to lung tumor-bearing mice resulted in gene expression in the new vasculature of primary tumors (86).

B. Treatment-responsive Promoters

Another strategy for cancer gene therapy involves regulating gene expression with another form of treatment, such as radiation. For example, the early growth response gene 1 (EGR-1) promoter, which is radiation inducible, has been used as a TSP for the specific expression of *lacZ* and *HSV-TK* in glioma and hepatocellular carcinoma cells. Radiation-induced transcription of EGR-1 in these cells was accomplished with rela-

tively low doses (87). Another approach for dynamically controlling promoter expression involves chemically inducible promoters. For example, a tetracycline-activated promoter can be used to regulate gene expression and subsequent protein production by giving oral tetracycline (88). Withdrawal of the drug rapidly abrogates gene expression.

V. DOUBLE-TARGETED ADENOVIRUSES

Transductional and transcriptional targeting can be combined to create double-targeted viruses. Conceivably, this approach could be synergistic with regard to safety and efficacy. Initial proof of concept was achieved by using a pulmonary vasculature specific promoter and a lung endothelium-targeted adaptor strategy (56). Impressively, the tumor-to-liver ratio of gene expression was increased 300,000-fold when both targeting modalities were used. Also, double targeting for ovarian cancer has been achieved in vitro and in vivo (89). Transductional targeting with a sCAR-fibritin-antiErbB2-sFv adapter was able to increase gene transfer to target cells while reducing transduction of nontarget cells. When combining transcriptional targeting with the SLPI promoter, an increase in selectivity was seen. Also, the transductional targeting increased the level of SLPI-mediated transgene expression in target cells, thereby compensating for the lower gene expression typically seen with TSPs.

VI. TRANSCRIPTIONAL AND TRANSDUCTIONAL TARGETING FOR CONDITIONALLY REPLICATING ADENOVIRUSES

Although nonreplicating first-generation Ad vectors have provided high in vitro and in vivo transduction rates and good safety data, clinical cancer trials have suggested that the single-agent antitumor effect may not be sufficient for all treatment approaches (18). Viruses that replicate and spread specifically inside the tumor have been suggested as a way to improve tumor penetration with an additional benefit of local amplification of effect. To this end, conditionally replicating adenoviruses (CRAds) have been explored. These viruses are genetically modified to take advantage of tumor-specific changes that allow preferential replication of the virus in target cells (19,90–94). The viral replication cycle causes oncolysis of the cell, resulting in the release of newly generated virions and subsequent infection of neighboring cells. Thus, the antitumor effect is not delivered with a transgene but by replication of the virus per se. In theory, the oncolytic process continues as long as target cells for the virus persist. There are two main ways to control viral replication. One method is the control of replication regulators, such as the viral early genes, with TSPs. The other method involves introduction of deletions in the viral genome that require specific cellular factors to compensate the effects of these deletions. Further, both approaches can be combined with the potential for increased specificity. Therefore, CRAds are by definition transcriptionally targeted agents.

Various promoters have been used to control viral replication (95–101), reviewed in (19). Typically, the TSP is placed to control expression of *E1A*, the crucial regulator of Ad replication, sometimes combined with other genes such as *E1B* or *E4*. An interesting concept is targeting CRAds to tumor vasculature (102). However, this strategy is more challenging to study preclinically, as animal models are unavailable – endothelial cells derive from the host in xenograft systems and murine cells do not support replication of human Ads. To further increase the oncolytic effect, transgenes for cytokines or prodrug-activating enzymes have been included in CRAds (103,104). This approach may also allow noninvasive imaging and abrogation of virus replication in case of toxicity.

Heretofore, two approaches have been used for creation of deletion-type CRAds. The first was ONYX-015 (initially reported as dl1520), which has two mutations in the gene coding for the E1B 55-kd protein (105,106). The purpose of this protein is binding and inactivation of p53 in infected cells, for induction of S-phase, which is required for virus replication. Thus, this virus should only replicate in cells with an aberrant p53-p14ARF pathway, a common feature in human tumors (107). Although this is still subject of debate, initial studies suggested that this agent replicates more effectively in tumor than in normal cells (108–111). Unfortunately, the function of E1B55kD is not limited to p53 binding, which causes inefficient replication compared with wild-type adenovirus (105,106,112).

The second group of deletion-mutant CRAds have a 24-bp deletion in the constant region (CR) 2 of *E1A* (113,114). This domain of the E1A protein is responsible for binding the retinoblastoma tumor suppressor/cell cycle regulator protein (Rb), thereby allowing Ad to induce S-phase entry. Therefore, viruses with this type of deletion have reduced ability to overcome the G1-S checkpoint and replicate efficiently only in cells where this interaction is not necessary (e.g., tumor cells defective in the *Rb-p16* pathway). Appropriately, this pathway seems to be inactive in most all human tumors (115). It has been shown that replication of CR2-deleted viruses is attenuated in nonproliferating normal cells (113,114). Importantly, abrogation of replication was also demonstrated when Rb was reintroduced into otherwise permissive cells (113). Ads with mutations in both CR 1 and 2 of *E1A* have also been found to replicate selectively in tumor cells, although increase in selectivity in comparison to just CR2-deleted CRAds has not yet been demonstrated (116–119).

Like Ad vectors, most published CRAds rely on CAR for entry into cells. Unfortunately, CAR levels are variable in many types of clinical cancers (10–13,31–36) (14,15,17). Nevertheless, even CRAds with wild-type tropism have shown evidence of clinical utility (120). These initial successes suggest that if efficiency of infection and specificity of replication of these agents could be enhanced, further improvements in clinical efficacy could be gained. This is corroborated by demonstration of the close association between infectivity and oncolytic potency (54,121,122). Consequently,

infectivity-enhanced CRAds have been constructed, with impressive preclinical efficacy. Ad5-Δ24RGD features an RGD-4C modification of the fiber (123), and displays similar oncolytic potency to wild-type virus in ovarian cancer cells (124). Further, this virus is able to replicate in ovarian cancer primary cell spheroids and results in significantly prolonged survival in an aggressive orthotopic ovarian cancer model (124).

A Δ24-based agent featuring the serotype 3 knob (Ad5/3-Δ24) was created (125). This agent demonstrated dramatic antitumor efficacy in ovarian cancer cell lines, primary tumor specimens, and orthotopic animal models of ovarian cancer. The first TSP-controlled infectivity-enhanced CRAd has recently been constructed and tested on ovarian and pancreatic cancer substrates [Masato Yamamoto, submitted; (126)]. Replicative specificity was achieved with a Cox-2 promoter controlling expression of *E1A*, whereas the fiber was modified with RGD-4C.

A major problem in assessing CRAd efficacy and safety preclinically, is the lack of an appropriate animal model. Human serotype Ads or CRAds do not replicate productively in commonly used animal models. Therefore, meaningful safety data are difficult to obtain, and efficacy data may be skewed due to deficient immune responses in xenograft models. Further, evaluation of host–virus interactions and their modulation has not been possible. This problem could be partially alleviated if a syngeneic CRAds could be developed for existing animal models of cancer (129).

VII. CLINICAL TRIALS WITH MODIFIED ADENOVIRUSES

No trials have yet been completed with targeted adenoviruses. Nevertheless, numerous trials are in progress. Specifically, an ovarian cancer trial using the Fab-FGF2 adapter for targeting an Ad coding for HSV-TK (and subsequent ganciclovir administration) to FGF receptors is in progress (Hemminki, personal communication). Modification of the fiber with RGD-4C has been used to create advanced generation vectors for human trials. RGDTKSSTR incorporates an HSV-TK cassette for cell killing and ganciclovir analog imaging, whereas the somatostatin receptor cassette can be used for somatostatin analog imaging. The agent achieved effective killing of ovarian cancer substrates including cell lines, primary tumor samples, and efficacy in murine models of human ovarian cancer (58,59). Also, noninvasive imaging was performed and found feasible even during ganciclovir treatment for a period of more than 2 weeks (58).

RGDTKSSTR was used to demonstrate an important aspect of fiber modified Ads. Neutralizing antibodies (NAbs) are the main component of the humoral immune system responsible for inhibition of Ad infection, and are present in a large proportion of the population. Further, NAb titers are quickly induced following treatment with Ad (127). Many NAbs are conformation sensitive, and when the fiber shape is changed slightly, such as when incorporating a targeting moiety, partial escape from NAbs can be seen (58,59,127,128). An ovarian cancer trial with RGDTKSSTR is in process (Hemminki, personal communication). A similar virus employing a cytosine deaminase prodrug-converting enzyme has been tested for treatment of head and neck cancer and a trial is pending (Hemminki, personal communication). The first transductionally targeted CRAd trial with Ad5-Δ24RGD has received National Cancer Institute funding and will soon start enrolling glioma and ovarian cancer patients (Hemminki, personal communication).

VIII. CONCLUSIONS

Ads are currently the most widely used platform for gene delivery, due to their unparalleled efficacy in gene expression in dividing and nondividing cells. They are particularly useful for treatment of cancer, where temporary gene expression and immunogenicity are acceptable or even beneficial. Nevertheless, unimpressive clinical results with first-generation Ads has led to construction of infectivity-enhanced, retargeted agents capable of more effective gene delivery to target organs while reducing side effects to nontarget tissues. Also, an important field is transductional improvement of conditionally replicating viruses, which may be required for realization of the full clinical potential of the approach. Combinations of transductional and transcriptional targeting approaches are likely to increase the feasibility of using Ads and CRAds for treatment of human disease.

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4

Helper-dependent Adenoviral Vectors for Gene Therapy

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I. INTRODUCTION

Gene therapy is the amelioration of disease through the use of nucleic acid. This broad definition reflects the large spectrum of diseases that can be potentially considered for treatment, as well as the many methods of introducing the nucleic acid into target cells, which may or may not be directly affected by the disease. This chapter focuses on a very specific area of gene therapy research—the development and application of helper-dependent adenoviral vectors (HDAds) (also referred to as gutless, gutted, mini, fully deleted, high-capacity, Δ, pseudo) for gene therapy. Successful gene therapy requires a gene transfer vector that is able to efficiently transduce the target cells in vivo and provide high-level and long-term transgene expression in the absence of toxicity. How HDAds have measured up to these expectations is reviewed in this chapter.

II. THE ADENOVIRUS

The adenovirus (Ad) has a nonenveloped icosohedral capsid containing a linear double-stranded DNA genome of ~30–40 kilobase (kb). Of the ~50 serotypes of human Ad, the most extensively characterized are serotypes 2 (Ad2) and 5 (Ad5) of subgroup C [reviewed in (1)]. The 36-kb genomes of Ad2 and Ad5 are flanked by inverted terminal repeats (ITRs), which are the only sequences required in *cis* for viral DNA replication. A *cis*-acting packaging signal, required for encapsidation of the genome, is located near the left ITR (relative to the conventional map of Ad). The Ad genome can be roughly divided into 2 sets of genes (Fig. 1): the early region genes, E1A, E1B, E2, E3, and E4, are expressed before DNA replication; and the late region genes, L1 to L5, are expressed to high levels after initiation of DNA replication. The E1A transcription unit encodes 2 major E1A proteins that are involved in transcriptional regulation of the virus and stimulation of the host cell to enter an S-phase-like state and is the first early region to be expressed during viral infection. The two major E1B proteins are necessary for blocking host mRNA transport, stimulating viral mRNA transport, and blocking E1A-induced apoptosis. The E2 region encodes proteins required for viral DNA replication and can be divided into two subregions; E2a encodes the 72-kD DNA-binding protein and E2b encodes the viral DNA polymerase and terminal protein precursor (pTP). The E3 region, which is dispensable for virus growth in cell culture, encodes at least 7 proteins, most of which are involved in host immune evasion. The E4 region encodes at least 6 proteins, some functioning to facilitate DNA replication, enhance late gene expression, and decrease host protein synthesis. The late region genes are expressed from a common major late promoter (MLP) and are generated by alternative splicing of a single transcript. Most of the late mRNAs encode virion structural proteins. In addition to the early and late region genes, 4 other small transcripts are also produced. The gene-encoding protein IX (pIX) is colinear with E1B but uses a different promoter and is expressed at an intermediate time, as is the pIVa2 gene. Other late transcripts include the RNA polymerase III-transcribed VA RNA I and II.

Virus infection is initiated through the Ad fiber protein binding to the primary coxsackie-adenovirus receptors (CAR)

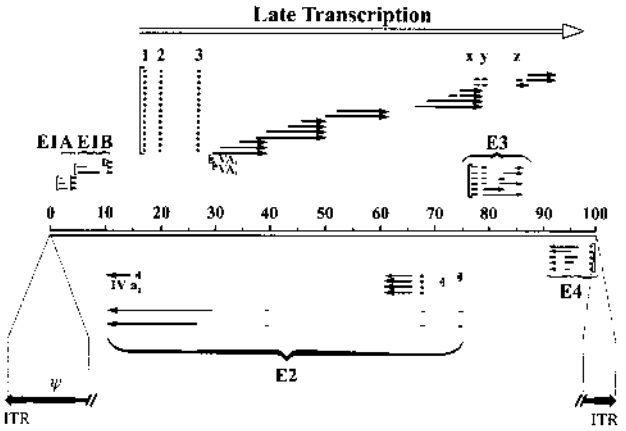

Figure 1 Transcription map of human adenovirus serotype 5. The 100 map unit (~36 kb) genome is divided into 4 early region transcription units, E1–E4, and 5 families of late mRNA, L1–L5, which are alternative splice products of a common late transcript expressed from the major late promoter located at 16 map units. Four smaller transcripts, pIX, IVa, and VA RNAs I and II, are also produced. The 103 bp inverted terminal repeats (ITRs) are located at the termini of the genome and are involved in viral DNA replication, and the packaging signal (ψ) located from nucleotides 190 to 380 at the left end is involved in packaging of the genome into virion capsids.

on the cell surface (2–4), followed by a secondary interaction between the virion penton base and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (5). The efficiency with which Ad binds and enters the cell is directly related to the level of primary and secondary receptors present on the cell surface (6,7). Penton–integrin interaction triggers Ad internalization by endocytosis, following which the virion escapes from the early endosome into the cytosol prior to lysosome formation (8,9). The virion is sequentially disassembled during translocation along the microtubule network toward the nucleus where the viral DNA is released into the nucleus (10). Once in the nucleus, viral DNA replication, beginning 6–8 h postinfection, and assembly of progeny virions occur. The entire life cycle takes about 24–36 h, generating about 10^4 virions per infected cell. Ads have never been implicated as a cause of malignant disease in their natural host and, in immunocompetent humans, most infections caused by the most common serotypes are relatively mild and self-limiting. For a more comprehensive discussion of adenoviruses, the reader is referred to the excellent review by Shenk (1).

III. FIRST-GENERATION ADENOVIRAL VECTORS

Ads are excellent mammalian gene transfer vectors due to their ability to efficiently infect a variety of quiescent and proliferating cell types from various species to direct high-level gene expression. Consequently, Ad vectors are extensively used as potential recombinant viral vaccines, for high-level protein production in cultured cells and for gene therapy (for reviews, see 11–15). First-generation Ad vectors (FGAds) typically have foreign DNA inserted in place of early region 1 (E1). E1-deleted vectors are replication deficient and are propagated in E1-complementing cells such as 293 (16). Typically, FGAds also have a deletion in the nonessential E3 region to maximize cloning capacity. The fundamental principle underlying all current methods of constructing FGAds is based on the discovery that up to 10% of Ad viral DNA molecules become circularized following infection of mammalian cells (17). This permitted cloning of the entire Ad genome as an infectious bacterial plasmid that could be manipulated with relative ease by standard molecular biology techniques. This Ad genomic plasmid could be stably propagated in *Escherichia coli* and was capable of generating infectious virus following transfection into permissive mammalian cells at efficiencies comparable to purified virion DNA. An excellent review by Danthinne and Imperiale (18) covers the plethora of methods for constructing FGAd. Although FGAd remain very useful for many applications, it has become clear that transgene expression in vivo is only transient. Several factors contribute to this, including strong innate and inflammatory responses to the vector (19,20), acute and chronic toxicity due to low level viral gene expression from the vector backbone (21), and generation of anti-Ad cytotoxic T-lymphocytes due to *de novo* viral gene expression (22–25) or processing of virion proteins (26). Although high-level transient transgene expression afforded by FGAd may be adequate, or even desirable, for many gene transfer and gene therapy applications, the toxicity and transient nature of expression kinetics renders these vectors unsuitable in cases where prolonged, stable expression is required

IV. MULTIPLY DELETED ADENOVIRAL VECTORS

Although the deletion of E1 in FGAds theoretically results in a replication-defective vector following transduction of cells devoid of trans-complementing E1 functions, it has become clear that this is not the case and that the E2, E3, and E4 promoters are active resulting in viral DNA replication and expression of the late viral genes, especially following high multiplicities of infection (27–30). Therefore, in an attempt to further attenuate Ad, additional deletions of essential viral genes have been pursued. Examples of these include deletion or mutation of the E2 or E4 regions in addition to E1. These multiply deleted Ad vectors (also referred to as second- or third-generation Ad vectors) are helper virus independent for propagation but require the generation of new producer cell lines that trans-complement the additional deletion. Despite the potential offered by these multiply deleted Ad vectors, viral coding sequences still remain and therefore so does the potential for their expression. The advantages of multiply de-

leted Ad over FGAd remain controversial because some studies show them to be superior in terms of toxicity and longevity of transgene expression (31–37), whereas others do not (21,38–41). Detailed discussion of multiply deleted Ad vectors is beyond the scope of this chapter; therefore, the reader is referred to an excellent and comprehensive review covering this subject by Parks and Amalfitano (42).

V. HELPER-DEPENDENT ADENOVIRAL VECTORS

A. Historical Perspective

In theory, the easiest way to completely eliminate the toxicity associated with viral gene expression is to delete all the viral coding sequences from the vector. Adenoviruses with large deletions of viral sequences were among the very first vectors reported. For example, in 1970, Lewis and Rowe (43) described a recombinant Ad in which essential viral genes were replaced with SV40 DNA. This replacement rendered the hybrids replication defective and they could only be propagated in the presence of a coinfecting wild-type helper Ad, which provided trans-complementation. A recombinant Ad was also described in 1981 by Deuring et al. (44), following repeated passages at high multiplicity infection of wild-type Ad serotype 12 through human KB cells. These hybrids were found to contain symmetrically duplicated human chromosomal DNA flanked by approximately 1 kb of DNA from the left end of the Ad genome. As in the case of the Ad-SV40 hybrids, these symmetrical recombinants (SYRECs) were replication defective and could only be propagated in the presence of a coinfecting helper Ad12 by trans-complementation. The SYRECs were maintained for years in this manner and could be partially purified from the helper virus by cesium chloride (CsCl) density equilibrium centrifugation, owing to differences in their genome sizes that bestowed different buoyant densities.

The defective helper virus-dependent Ad-SV40 and SYREC hybrids demonstrated the possibility of generating vectors completely devoid of Ad coding sequences. In principle, only ~500 bp of *cis*-acting Ad sequences necessary for DNA replication (ITR) and encapsidation (ψ) are required for propagation of these HDAds in the presence of a coinfecting helper virus. The advantages of HDAds are considerable: like first-generation (FG) and multiply deleted Ads, HDAds would retain the ability to efficiently transduce a wide variety of cell types from numerous species in a cell-cycle-independent manner. However, unlike FG and multiply deleted Ads, deletion of all viral coding sequences would drastically reduce vector-mediated toxicity and significantly prolong the duration of transgene expression as described in detail below.

B. Early Systems for Generating HDAds

Although, as mentioned above, the first Ad vectors were defective, helper-dependent viruses, with the development of E1-complementing 293 cells vector development focused on FGAd (E1 deleted) vectors. These were much easier to isolate and propagate than HDAds. The possibility of using HDAds for gene transfer was reexamined in studies reported by Mitani et al. (45). These investigators used a β-galactosidase-neomycin fusion gene to replace 7.3 kb of essential Ad sequences in an Ad genomic plasmid. Although this modification did not remove all the viral coding sequences, it did render the recombinant defective and helper dependent. This vector was rescued by cotransfection of 293 cells with purified Ad2 DNA, which provided helper functions. One percent to 5% of the resulting plaques turned blue following X-gal staining, indicating rescue of the recombinant virus. Blue plaque isolates were expanded by serial propagation on 293 cells and finally purified by CsCl ultracentrifugation. Fractionation through the gradient resulted in partial purification of the vector from the helper virus due to the difference in their buoyant densities. Significantly, the ability of the vector to transduce cells in vitro and express the reporter gene was demonstrated. However, the yield of vector was quite low. Furthermore, helper virus contamination remained rather high, at about 200- to 500-fold greater than the vector. In addition, the genome of the vector had undergone rearrangement.

Using a different strategy, Fisher et al. (46) constructed a plasmid bearing a 5.5-kb HDAd genome containing the Ad 5′ ITR and packaging signal, LacZ reporter gene, and 3′ ITR. 293 Cells were infected with a FGAd to serve as a helper virus and subsequently transfected with the HDAd plasmid DNA. The HDAd was amplified by serial coinfections and finally purified by CsCl ultracentrifugation. Using this method, partial purification of the vector could be achieved, but with the helper virus still in 10- to 100-fold excess. In addition, considerable genomic rearrangements in the form of concatemerization of the vector were observed, which were likely due to the small size of the vector's genome (47). Nevertheless, the vector was demonstrated to be capable of transducing cells in vitro. Using this strategy, a vector bearing cystic fibrosis transmembrane conductance regulator (CFTR) (46) and dystophin (48) expression cassettes was generated and shown to transduce cells in vitro.

Another strategy for generating HDAds was reported by Kumar-Singh and Chamberlain (49). In this system, 293 cells were cotransfected with a plasmid bearing the HDAd genome and purified Ad DNA to provide helper functions. The HDAd contained a LacZ reporter gene and dystrophin cDNA, whereas the helper virus contained the alkaline phosphatase (AP) reporter gene. The vector was amplified by serial propagation and purified by CsCl ultracentrifugation. This resulted in a final vector preparation with 4% helper virus contamination, as determined by AP:LacZ ratio. Importantly, this vector was capable of transducing myogenic cell cultures and express dystrophin in myotubes.

These early systems showed that HDAds could indeed be generated and could transduce a variety of target cells in vitro to direct transgene expression. However, they also emphasized the need to further improve production strategies because the high levels of helper virus contamination, low vector yield and, in many cases, vector genome rearrangement were clearly obstacles that needed to be addressed before the full

potential of HDAd could be realized. In particular, relying solely on physical separation between the vector and the helper by CsCl ultracentrifugation was clearly inadequate to achieve the desired vector purities. Therefore, strategies to preferentially inhibit helper virus propagation, while not affecting its ability to trans-complement the HDAd, were required.

One system designed to specifically address preferential inhibition of helper virus propagation was reported in 1996 by Kochanek et al. (50). This strategy was based on early studies in Ad packaging by Gräble and Hearing (51), which showed that deletion of 91 bp from the packaging signal severely reduced, but did not abolish, encapsidation of the Ad genome into virions while not affecting viral DNA replication. More important, Gräble and Hearing observed a competition for packaging in cells coinfected with 2 Ads: one containing a wild-type packaging signal and the other having a mutant packaging signal. The former was packaged preferentially, almost to the exclusion of the latter. Taking advantage of these observations, Kochanek et al. deleted this 91-bp deletion from the Ad packaging signal of the helper viral genome, thus impairing its ability to be packaged but not affecting its ability to replicate and thus trans-complement the HDAd genome (50). In addition, because the HDAd genome contained the wild-type Ad packaging signal, it would be preferentially packaged over the helper viral genome. The combination of this strategy in conjunction with CsCl ultracentrifugation resulted in a final vector preparation with 1% helper virus contamination. A similar strategy, but using a different packaging signal mutation, was employed by Alemany et al. (52) to generate HDAds. These strategies represented a significant improvement over the previous methods in terms of lower levels of helper virus contamination. However, 1% helper virus contamination would still likely be too high for human use, especially if high doses of HDAds were required. Furthermore, the 91-bp packaging signal deletion resulted in 90-fold reduction in yield of helper virus. From a practical standpoint, this would render production of helper virus stocks problematic because large amounts would be needed to produce the considerable quantities of HDAd needed for clinical applications.

Another system designed to specifically address propagation of the helper virus was reported by Liber et al. (53). This system took advantage of the bacteriophage P1 Cre recombinase, which catalyzes site-specific recombination between 34 bp loxP. In this method, an FGAd was engineered to contain 2 parallel loxP sites, one immediately downstream of transgene expression cassette used to replace E1 and the other in E3. Following infection of 293 cells expressing Cre, 25 kb of Ad sequences was excised from the FGAd, leaving behind a 9-kb HDAd. Thus, the unrecombined FGAd acted as the helper virus for the propagation of a 9-kb HDAd. Unfortunately, the small HDAd was unstable in vivo, providing limited duration transgene expression for reasons that are still not fully understood. Furthermore, the vector still retained Ad coding sequences and, because the HDAd originated from an FGAd, had limited cloning capacity. Clearly, further improvements were needed if HDAd were to progress into clinical applications.

C. The Cre/loxP System for Generating HDAds

The first efficient and currently most widely used method for generating HDAd was the Cre/loxP system developed by Graham and coworkers in 1996 (54) (Fig. 2). In most versions of this system, the HDAd genome is first constructed in a bacterial plasmid. Minimally, the HDAd genome contains the expression cassette of interest and ~500 bp of *cis*-acting Ad sequences necessary for vector DNA replication (ITRs) and packaging (ψ). As described in detail in Section V.E, inclusion of stuffer DNA in the HDAd genome is often required for efficient packaging. To rescue the HDAd (i.e., to convert the ''plasmid form'' of the HDAd genome into the ''viral form''), the plasmid is first digested with the appropriate restriction enzyme to liberate the HDAd genome from the bacterial plasmid sequences. 293 Cells expressing Cre are then transfected with the linearized HDAd genome and subsequently infected with the helper virus. The helper virus bears a packaging signal flanked by loxP sites and thus, following infection of 293Cre cells, the packaging signal is excised from the helper viral genome by Cre-mediated site-specific recombination between the loxP sites. This renders the helper viral genome unpackagable, but still able to undergo DNA replication and thus trans-complement the replication and encapsidation of the HDAd genome. The titer of the HDAd is increased by serial coinfection of 293Cre cells with the HDAd and the helper virus (Fig. 3). Maximum HDAd titer of $\sim 10^8$ infectious particles/mL (~1000–2000 infectious particles/producer cell) is usually obtained by the third serial passage (Fig. 3)(55). Typically, the level of helper virus contamination is ($\leq$1%, as determined by Southern blot hybridization analysis, before vector purification by CsCl ultracentrifugation (55) and, provided that the genome sizes of the vector and helper are sufficiently different, purities of $\leq$0.1% helper virus contamination can be achieved following CsCl ultracentrifugation as determined by Southern blot hybridization or quantitative real-time polymerase chain reaction (PCR) (56). Detailed methodologies for producing HDAd using the Cre/loxP system are described elsewhere (57). A similar system was subsequently reported by Hardy et al. (58).

D. The FLP/frt System for Generating HDAds

Based on the Cre/loxP strategy, systems were later developed using the yeast site-specific recombinase FLP, which catalyzes recombination between 34-bp frt sites (55). In this case, the packaging signal in the helper viral genome is flanked by frt sites so that, following infection of 293 cells stably expressing FLP, the packaging signal is excised, rendering the helper viral genome unpackagable but still able to replicate and trans-complement propagation of the coinfecting HDAd. This system was shown, by direct comparison, to be compara-

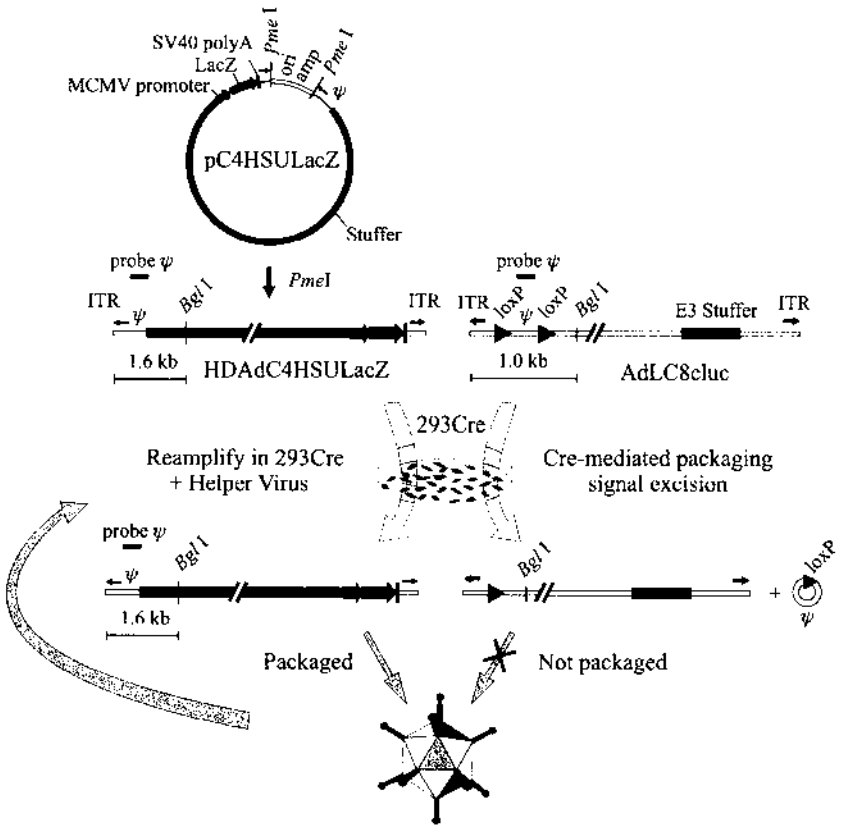

Figure 2 The Cre/loxP system for generating HDAds. The HDAd contains only ~500 bp of *cis*-acting Ad sequences required for DNA replication (ITRs) and packaging (ψ), the remainder of the genome consists of the desired transgene, in this case, a LacZ expression cassette, and non-Ad ''stuffer'' sequences. The HDAd genome is constructed as a bacterial plasmid (pC4HSULacZ in this example) and is liberated by restriction enzyme digestion (e.g., *Pme*I). To rescue the HDAd (HDAdC4HSULacZ), the liberated genome is transfected into 293Cre cells and infected with a helper virus bearing a packaging signal (ψ) flanked by loxP sites (e.g., AdLC8cluc,54). The helper viral genome also contains a stuffer sequence in E3 to prevent the formation of RCA in 293-derived cells. Cre-mediated excision of ψ renders the helper virus genome unpackagable, but still able to replicate and provide all the necessary *trans*-acting factors for propagation of the HDAd. The titer of the HDAd is increased by serial coinfections of 293Cre cells with the HDAd and the helper virus. Shown for the HDAd and the helper virus are the relevant BglI sites and the corresponding DNA fragment sizes, as well as the location of the packaging signal probe (probe ψ) used for Southern blot hybridization analysis illustrated in Fig. 3.

ble to the Cre/loxP system in terms of efficiency of HDAd amplification and low helper virus contamination levels (55). A similar FLP-based system for generating HDAds was also reported by Umana et al. (59). The availability of 2 alternative systems for generating HDAd should expand the utility of HDAds by permitting use of recombinase-activated ''molecular switches'' (60) in which one recombinase can inhibit helper virus propagation while the other regulates transgene expression. Furthermore, it has been suggested that the 2 site-specific recombinase systems may be combined to further reduce the level of helper virus contamination, perhaps by flanking the packaging signal with both loxP and frt sites and using producer cells that express both Cre and FLP (55).

E. Characteristics of the Helper-dependent Vector

In addition to the minimal *cis*-acting Ad sequences required for DNA replication and encapsidation, Sandig et al. (56) showed that inclusion of a small segment of noncoding Ad sequence from the E4 region adjacent to the right ITR increases vector yields, possibly by enhancing packaging of the HDAd DNA. Early studies of Ad have established a maximum packaging capacity of 105% of the wild-type genome (~37.8 kb) (61). Subsequently, using the Cre/loxP system for generating HDAds, Parks et al. (47) established that the minimum genome size for efficient packaging into Ad virions was 27 kb.

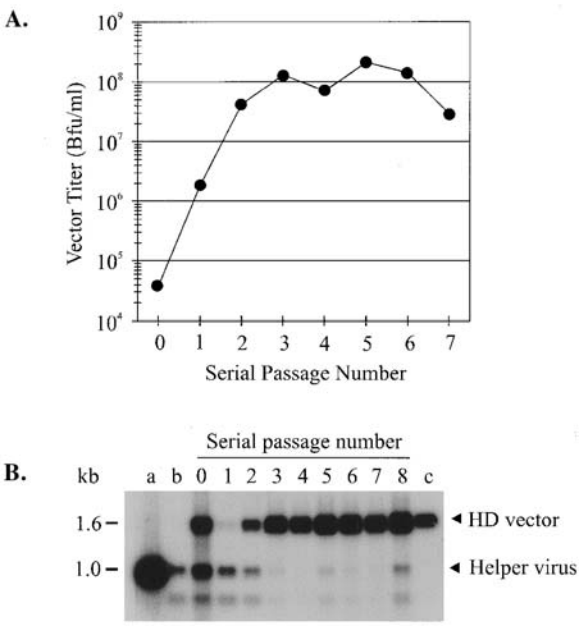

Figure 3 Amplification of HDAd. (A) Amplification of HDAdC4HSULacZ by serial coinfections of 293Cre cells with the HDAd and the helper virus. HDAdC4HSULacZ contains a LacZ reporter gene (Fig. 2). Therefore, the amount of HDAd produced at each serial passage can be determined by X-gal staining and is expressed as blue-forming units (bfu)/mL. (B) Southern blot hybridization analysis of HDAd amplification from (A). Total DNA was extracted from the coinfected cells at each serial passage, digested with BglI, and analyzed with a packaging signal probe (see Fig. 2 for details). The amount of HDAd DNA (1.6-kb band) increases from passage 0 to a peak by passage 3, consistent with amount of infectious vector produced as shown in (A), whereas the amount of packagable helper viral DNA (1.0-kb band) remains low. Lane a, total DNA extracted from helper virus-infected 293 cells. Lane b, total DNA extracted from helper virus-infected 293Cre cells. Lane c, pC4HSULacZ digested with BglI and *Pme*I. (Adapted from Ref. 55.)

Vector genome sizes above the maximum packaging capacity were not efficiently packaged, if at all. Vector genome sizes below 27 kb were inefficiently packaged and frequently underwent DNA rearrangements to produce genomes closer to that of wt viruses (approximately 36 kb) (46,47,62). Therefore, the size of the vector is an important consideration: for efficient packaging and stability, the vector genome should be between ~75% (>27 kb) (47) and 105% (<37.8 kb) (61) of wild-type. Because the minimal Ad *cis*-acting sequences and the transgene of interest are usually well below the minimal size required for efficient packaging, the vector must often include ''stuffer'' DNA. The choice of stuffer DNA is important with regard to vector stability, replication efficiency and in vivo performance (56,63,64). Although it remains unclear what constitutes a good stuffer, in general, noncoding eukaryotic DNA has been preferred, whereas repetitive elements and unnecessary homology with the helper virus should be avoided to ensure vector stability and prolonged transgene expression.

In addition to the absolute size of the HDAd, its relative size compared with the helper virus has an impact on the production of (nearly) helper-free vectors. This is because Cre-mediated selection against the helper virus, although efficient, is not absolute (65) (see Section V.G). If the genome size of the HDAd is sufficiently different from that of the helper virus then the 2 species can be physically separated by CsCl ultracentrifugation due to their different buoyant densities. HDAds between 28 and 31 kb have proven ideal because they are efficiently packaged and replicated, as well as easily separated from residual helper viruses (35–37 kb) following CsCl ultracentifugation.

It is also becoming clear that the nature of the transgene has a significant influence on the degree and duration of transgene expression. Specifically, transgenes in their native genomic context have consistently been demonstrated to be superior to their cDNA counterparts with respect to level and duration of expression (64,66,67). This is likely due to a more natural regulation of gene expression. In this regard, because of the large cloning capacity of 37 kb, HDAds offer the advantage of potentially transferring many genes in their genomic context, whereas other vectors (e.g., FG and multiply deleted Ads, retroviral, lentiviral, or adeno-associated viral vectors) cannot due to their limited cloning capacity. In the case of cDNA, expression from a tissue-specific promoter was found to be superior to nonspecific promoters in terms of toxicity and duration of expression (64,68). This has been attributed to a reduction of transgene expression in transduced antigen-presenting cells, following systemic delivery of vectors that carry expression cassettes under the control of tissue-specific promoters (68). Expression from tissue-specific promoters has been demonstrated to be more ''tissue specific'' within the context of an HDAd than a FGAd (69). This may be due to the influence of Ad sequences from the FGAd backbone on transgene expression specificity, which does not occur with HDAds (69). In addition, use of tissue-specific promoters may result in more robust amplification of the HDAd because high-level transgene expression in the producer cells may negatively impact viral replication during serial coinfections, especially if the transgene product is toxic to the producer cells.

Titration of FG and multiply deleted Ads is straightforward. These vectors can form plaques on monolayers of the appropriate complementing cell lines in a standard plaque assay. The infectious titer can then be determined by enumerating the plaques and expressed as the number of plaque-forming units (pfu)/mL. In addition to infectious titer, the virion particle concentration, based on DNA content, can be readily obtained by measuring the optical density at 260 nm (OD_{260}) of the vector preparation. Comparison of the particle: infectious unit ratio provides a measure of the infectivity of the vector preparation and current Food and Drug Administration standards require a ratio of <30:1 for FG vectors used in

clinical trials (70). Like FG and multiply deleted Ads, the physical titer of HDAds can be readily obtained by measuring OD_{260}. Measurements of infectious titers are, however, not as straightforward because, unlike FG and multiply deleted Ads, HDAds cannot form plaques on cell monolayers, thus precluding infectious titer determination by standard plaque assays. If the HDAd contains a transgene that can serve as a reporter, such as LacZ, an infectious titer can be obtained (see, for example, Fig. 3A). However, this is not the case with the majority of therapeutic HDAds, thus rendering the infectivity of HDAd preparations difficult to ascertain. Kreppel et al. (71) developed a DNA-based method of measuring the infectivity of HDAds that assays ability of the vector to deliver its genome into target cells. However, it is important to note that this method measures only cellular entry of the vector DNA, whereas infectious titer assays for FG and multiply deleted Ads also depend on viral gene expression (resulting in plaque formation or reporter gene product) following entry of the genome into the nucleus and are thus more stringent.

F. Characteristics of the Helper Virus

The most commonly used helper virus is a serotype 5, FGAd (E1 deleted) with its packaging signal flanked by loxP sites (Fig. 2). As with all FGAds propagated in 293 or 293-derived cells, the potential exists for the generation of replication-competent Ad (RCA; $E1^+$) as a consequence of homologous recombination between the helper virus and the Ad sequences present in 293 cells. To prevent the formation of RCA, a ''stuffer'' sequence was inserted into the E3 region to render any $E1^+$ recombinants too large to be packaged (54). The length of the E3 stuffer is such that the total size of the helper virus genome is $<105\%$ of wild-type, but $>105\%$ following homologous recombination with Ad sequences in the 293 cells. As of this writing, the emergence of RCA has yet to be reported using helper viruses with an E3 stuffer. In contrast, RCA is readily detected using helper viruses without an E3 stuffer when propagated in 293 and 293-derived cells (41,54). The choice of sequence used as stuffer in the E3 region may be important as it has been observed that some E3 inserts result in poor virus propagation, perhaps due to interference with fiber expression (Authors' unpublished data). Although it remains unclear what sequences constitute a good E3 stuffer, in general, noncoding sequences with no homology to the HDAd would be preferred. A number of sequences have been found to be stable and not to adversely affect virus propagation, including bacterial plasmid sequences, bacteriophage λ sequences, and human DNA (54–56,59,72). The use of other E1-complementing cell lines engineered to preclude the formation of RCA is another option (73,74). Such cell lines have been used for the production of RCA-free FGAd, but there are not yet any reported of the development of Cre-expressing derivatives and their use for the production of HDAd.

One strategy to further enhance safety is to attenuate the helper virus to reduce its toxicity. To this end, a helper virus with a deletion of E2a was developed by Zhou et al. (75). E2a encodes the Ad 72-kD DNA-binding protein, which is essential for viral DNA replication. In theory, the E2a-deleted contaminating helper viruses would be unable to undergo viral DNA replication and thus would be unable to express the late Ad structural proteins, which are responsible for cytotoxicity and induction of cellular immunity. However, whether this modification truly improves safety is questionable, considering some studies have shown that the immune response and toxicity elicited against Ad is the same regardless of whether the vector has a defective E2a (21,41) (also see Section IV).

Because the HDAd genome does not integrate into the host chromosomes, but rather presumably remains episomal, it is likely that transgene expression will not be permanent. If transgene expression fades over time, it would be desirable to simply readminister the vector. Unfortunately, this simplistic approach is not possible because the initial administration elicits immunity in the form of neutralizing anti-Ad antibody, which renders subsequent readministrations ineffective. One strategy, known as ''serotype switching'' may help to overcome this problem. In addition to the Ad serotype 5-based helper virus, Parks et al. have generated a serotype 2 helper virus (72). Helper viruses based on serotypes 1 and 6 have also been generated (Authors' unpublished data). Therefore, genetically identical HDAds of different serotypes can by generated simply by changing the serotype of the helper virus used for vector amplification. Parks et al. demonstrated that mice injected with a serotype 2 HDAd produced serotype 2 neutralizing antibodies, which prevented successful transduction with the same serotype HDAd (72) but did not prevent successful transduction following administration of a serotype 5 HDAd. Successful readministration of HDAd of alternative serotypes has also been demonstrated by Kim et al. (66) (see Section VI.A). As discussed in Section II, there are ~50 human serotypes of Ad. Therefore, it may be possible to create a panel of different serotype helper viruses and use these to generate different serotype but genetically identical HDAds. These HDAds could then be given sequentially every 2 to 3 years when transgene expression wanes from the previous vector administration. Because of the large number of Ad serotypes, this could theoretically continue for the lifetime of the patient, although development of the many helper viruses needed for this approach might prove challenging.

G. Helper Virus Contamination

Currently, the final purity of HDAd preparations is dependent on two enrichment steps. The first is Cre-mediated packaging signal excision during vector amplification. This alone results in about $\leq 1\%$ helper virus contamination (65). If the genome sizes of the HDAd and the helper virus are sufficiently different, further enrichment can be obtained by CsCl ultracentrifugation, which can reduce contamination levels to as low as 0.1% or less. Although the final level of helper virus contamination is quite low, further reduction is desirable to minimize any potential toxicity associated with the helper virus, especially in therapies where very high doses of the HDAd may be required. Furthermore, in those cases where the genome sizes of the HDAd and helper virus are unavoidably similar,

physical separation of the 2 species by CsCl ultracentrifugation will not be possible and contamination levels of ≤0.1% will likely be unachievable. Finally, CsCl ultracentrifugation is impractical for large-scale clinical grade vector production. The source of the residual contaminating helper virus that persists during HDAd propagation using the Cre/loxP has been investigated in detail (65). That study revealed that the contaminating helper virus has escaped Cre-mediated packaging signal excision and propagated. Detailed investigations revealed that this was not due to acquisition of Cre-resistant mutations or to the reverse Cre reaction, which would reinsert the excised packaging signal. Nor was it due to inaccessibility of a fraction of the helper viral DNA to Cre. Rather, the results revealed that incomplete packaging signal excision was the result of Cre levels being limiting in the producer cells due to low endogenous levels. Further exacerbating this problem may be Ad-mediated host cell shut off, a well-documented phenomenon in which synthesis of cellular protein is inhibited as a consequence of Ad infection [reviewed in (76)]. The results of this study suggested that further reduction in helper virus contamination may be achieved by increasing the amount of Cre in the producer cells. However, given that very high, constitutive levels of Cre expression have been reported to be cytotoxic in various cell lines, including 293 (77), this strategy may be difficult to achieve.

It is important to ascertain the level of helper virus contamination accurately because high levels of helper virus contamination may affect experimental results and compromise safety. Knowing the level of helper virus contamination also allows for meaningful comparisons between different studies. A variety of methods for determining helper virus contamination have been reported, all of which can be divided into 2 basic categories. The first is based on determining the infectious titer of the contaminating helper virus in an HDAd preparation. These methods included plaque assay on 293 cells (or 293-derived cells) or infectious unit assay, based on the presence of a reporter transgene in the helper virus. Using these infectious titer assays, the level of helper virus contamination is often reported as a percent or a ratio of infectious helper virus in the vector preparation. However, these methods suffer from numerous shortcomings. First, the units of measurement for the HDAd and helper virus are not the same and thus not directly comparable, making it difficult to accurately determine the level of helper virus contamination. Second, it has been shown that infectious assays are 10- to 50-fold less sensitive than DNA-based assays described below, thus grossly underestimating the true level of contamination, and may be incapable of detecting very low levels of helper virus contamination (41,71). Alternative methods of determining helper virus contamination use direct measurement of the amount of HDAd and helper viral DNA in a vector preparation. This has been accomplished by standard Southern blot hybridization analysis (55,71) or quantitative real-time PCR assays (41,56,59). Not only are these methods more sensitive and reliable, but also the unit of measurement is the same for both the HDAd and the helper virus therefore allowing the level of helper virus contamination to be expressed simply as a percentage of the total DNA [% helper contamination = helper DNA/(vector DNA + helper DNA)]. The reader should keep the distinction between the 2 methods of determining helper virus contamination levels in mind when critically reading primary publications. In general, most studies published prior to the year 2000 used infectious titer assays, whereas most (but not all) studies published after the year 2000 used DNA-based methods to determine helper virus contamination levels.

VI. IN VIVO STUDIES

As of this writing, numerous examples of in vivo gene transfer using HDAds have been reported. This chapter does not provide a comprehensive review of these studies; instead, examples of particular significance or interest are described. In general, all these studies demonstrate that compared with FG and multiply deleted Ads, HDAds are significantly less toxic and can permit prolonged transgene expression in vivo.

A. Transduction of the Liver

The liver is very attractive target for gene therapy. The fenestrated structure of its endothelium permits exposure of the parenchymal cells to intravenously delivered vector and secretion of vector-encoded therapeutic proteins into the circulation for systemic delivery. Ads are particularly attractive vectors for liver-directed gene therapy because of their efficiency at transducing hepatocytes following intravenous injection.

α_1-Antitrypsin antagonizes neutrophilic elastase and is abundantly expressed in hepatocytes and at a lower level in macrophages. α_1-Antitrypsin-deficient patients have shortened life expectancies due to emphysema. The utility of HDAds for liver-directed gene transfer was demonstrated in a series of studies using a HDAd containing the 19-kb genomic human α_1-antitrypsin (hAAT) locus (AdSTK109). In the first of these studies, immunocompetent C57BL/6J mice, which do not generate anti-hAAT antibodies, were intravenously injected with 2×10^{10} particles of AdSTK109 (67). Serum hAAT levels reached a plateau of ~50 μg/mL 3 weeks postinjection and were sustained for the duration of the 10-month experiment. In contrast, in C57BL/6J mice injected with 2×10^{10} particles AdhAATΔE1, a FGAd vector bearing the hAAT cDNA, hAAT concentrations reached a peak of 2 μg/mL 3 days postinjection, followed by a slow decline over 10 months to less than 10% of the peak levels. The superior expression levels induced by AdSTK109 compared with AdhAATΔE1 were attributed to the more favorable genomic context of the hAAT in the HDAd compared with the cDNA inserted in the FGAd. The duration of hAAT expression correlated with persistence of vector genome in the liver. Specifically, a total of 65% of the FGAd genome was lost between 3 days and 12 weeks compared with a loss of only 30% in the case of AdSTK109, with only a 6% loss at 6 and 12 weeks. Histopathological examination of livers from C57BL/6J mice injected with AdSTK109 revealed normal morphology between 3 days

and 12 weeks postinjection, whereas significant hepatotoxicity was observed from 6 to 12 weeks in the livers of mice injected with AdhAATΔE1. To further examine the issue of hepatotoxicity, *Rag1*-immunodeficient mice were injected with AdSTK109 or AdhAATΔE1. As with C57BL/6J mice, no evidence of liver toxicity was associated with AdSTK109. In contrast, hepatotoxicity was observed in AdhAATΔE1-injected *Rag1* mice, similar to that observed in C57BL/6J mice. These results suggested that the observed hepatotoxicity resulted from direct cytotoxic affect of the viral proteins expressed from the AdhAATΔE1 backbone, instead of toxicity due to induction of a cytotoxic T lymphocyte (CTL) response because *Rag1* mice do not produce CTLs (67).

Increasing the dose of AdSTK109 to 1.1×10^{11} particles resulted in 1 mg/mL hAAT in the serum, which is close to the normal human range (1.3 mg/mL) (78). Further increases in vector dose to 3.2×10^{11} particles resulted in supraphysiological levels of 5–6 mg/mL, which were sustained for the duration of the experiment (8 weeks). In contrast, injection of identical doses of AdhAATΔE1 resulted in only transient hAAT expression. Clinically relevant markers of liver toxicity (ALT, alanine aminotransferase; AST, aspartate aminotransferase; and AP, alkaline) remained within the normal range in mice injected with AdSTK109 throughout the observation period, beginning 3 days to 8 weeks postinjection. In contrast, these markers were significantly elevated in mice injected with AdhAATΔE1 for at least 6 weeks. Histopathological studies performed 5 days postinjection with the highest dose of AdSTK109 (3.2×10^{11} particles) revealed only mild signs of liver injury, including minor evidence of inflammation as quantitated by neutrophil infiltration. In contrast, extensive liver injury was observed at the highest dose of AdhAATΔE1 (3.2×10^{11} particles), including cell degeneration, cell necrosis, and abundant inflammation. Morphological changes were also observed in the spleen at the highest dose, including large, expanded lymphoid follicles with active germinal centers and increased extramedullary hematopoiesis. These splenic changes were more pronounced in mice injected with AdhAATΔE1. Southern blot analyses on liver DNA revealed that AdSTK109 DNA did not decline significantly for the duration of the experiment. In contrast, the amount of AdhAATΔE1 DNA in the liver at 8 weeks was significantly reduced compared with 5 days in mice injected with high dose, consistent with the observed decline in hAAT expression.

To evaluate the utility of HDAds in a large animal model, three baboons were intravenously injected with $3.3–3.9 \times 10^{11}$ particles/kg of AdSTK109 (79). hAAT expression persisted for more than 1 year in 2 of the 3 animals (Fig. 4). Maximum levels of serum hAAT of 3–4 mg/mL were reached 3 to 4 weeks postinjection in these 2 baboons and slowly declined to 8% and 19% of the highest levels after 24 and 16 months, respectively. The slow decline in hAAT expression was attributed to the fact that the baboons were young (7.5 and 9 months old) when injected and that the decrease in hAAT concentrations was correlative to the growth of the animals. The third baboon injected with AdSTK109 had significantly lower levels of serum hAAT, which rapidly declined

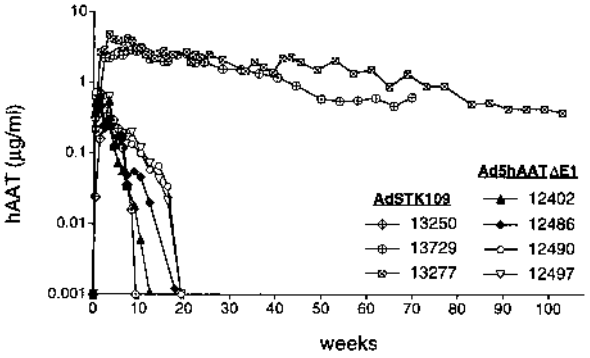

Figure 4 Serum levels of hAAT in baboons following intravenous administration of the HDAd AdSTK109 or the FGAd Ad5-hAATΔE1. Baboons 12402 and 12486 were injected with 6.2×10^{11} particles/kg of Ad5hAATΔE1. Baboons 12490 and 12497 were injected with 1.4×10^{12} particles/kg of Ad5hAATΔE1. Baboons 13250, 13729, and 13277 were injected with 3.3×10^{11} particles/kg, 3.9×10^{11} particles/kg, and 3.6×10^{11}particles/kg, respectively, of AdSTK109. (From Ref. 79, copyright 1999 National Academy of Sciences, USA.)

to undetectable levels after 2 months (Fig. 4). This baboon had generated anti-hAAT antibodies, thus accounting for the low level and rapid loss of serum hAAT. No abnormalities in blood cell counts and chemistries were observed in these 3 baboons at any time, starting 3 days postinjection. In contrast to baboons injected with AdSTK109, hAAT expression lasted only 3 to 5 months in all 4 baboons injected with AdhAATΔE1 (Fig. 4). This was shown not to be due to the generation of anti-hAAT antibodies but was attributed to the generation of a cellular immune response against viral proteins expressed from the vector backbone, resulting in the elimination of vector-transduced hepatocytes. These early experiments convincingly demonstrated that HDAd were superior to FGAd with respect to duration of transgene expression and hepatotoxicity in mice and, significantly, in a nonhuman primate.

Leptin is a potent modulator of weight and food intake. Leptin deficient *ob*/*ob* mice gain considerable weight (~70 g) compared with lean littermates (~28 g) at 8 to 12 weeks of age. Morsy et al. (62) compared HDAd with FGAd, with respect to safety and efficacy, for leptin gene therapy in *ob*/*ob* mice. Intravenous injection of $1–2 \times 10^{11}$ particles of a FG vector encoding murine leptin (Ad-leptin) into *ob*/*ob* mice resulted in an increase in serum leptin levels for only the first 4 days, returning to baseline levels 10 days postinjection (Fig. 5A). Increased leptin levels were associated with transient weight loss of ~25% followed by weight gain 2 weeks after treatment (Fig. 5B). In contrast, *ob*/*ob* mice injected with an HDAd-encoding leptin (HD-leptin) resulted in about 2-fold higher serum levels of leptin up to ~15 days postinjection (Fig. 5A). However, expression was transient and gradually returned to baseline levels 40 days postinjection. Rapid weight loss to levels approaching that of normal lean mice (>60%

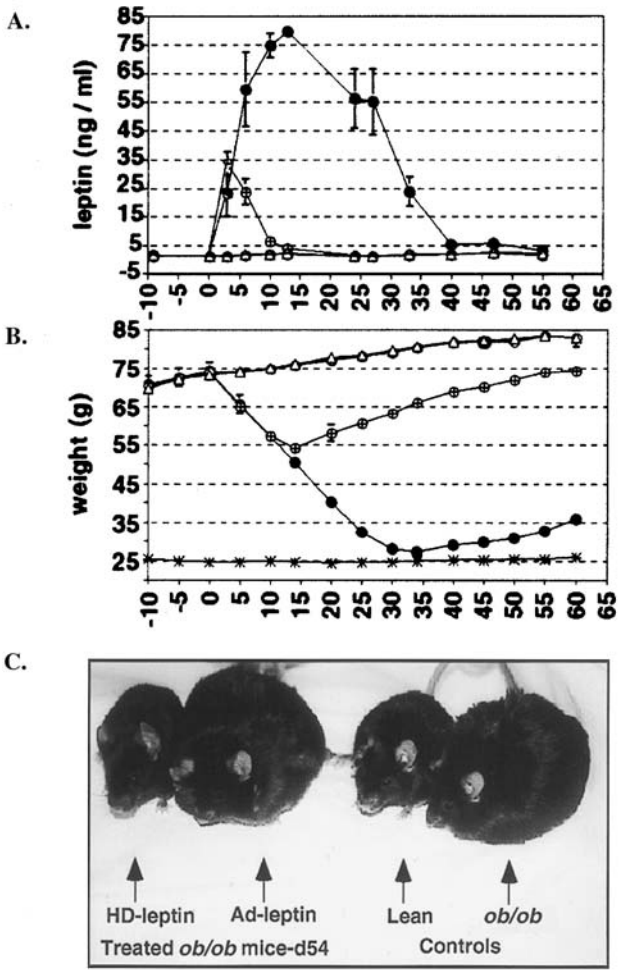

Figure 5 Phenotypic correction of leptin-deficient *ob*/*ob* mice with HDAd-mediated gene therapy. (A) Serum leptin levels and (B) weights of *ob*/*ob* mice intravenously injected with HD-leptin (black circle), Ad-leptin (crossed circle), or uninjected controls (white triangles). Weights of normal lean mice are shown in (B) as ∗. (C) Phenotypic correction of *ob*/*ob* mice. On the left is an *ob*/*ob* mouse treated with HD-leptin, next to an *ob*/*ob* littermate treated with Ad-leptin at 54 days postinjection. Control normal lean mouse and untreated *ob*/*ob* mouse are shown to the right for comparison. See the color insert for a color version of this figure. (Modified from Ref. 62, copyright 1998 National Academy of Sciences, USA.)

weight reduction) was observed in the HD-leptin-treated animals by 1 month, but the loss was only maintained for 6 to 7 weeks postinjection (Figs. 5B and 5C). Analogous to the case of hAAT in 1 baboon, loss of leptin in both Ad-leptin and HD-leptin mice correlated with the development of antileptin antibodies. This is undoubtedly due to the fact that *ob*/*ob* mice are naive to leptin. Hepatotoxicity was compared in mice treated with HD-leptin and Ad-leptin 1, 2, and 4 weeks postinjection. Pronounced liver toxicity, as measured by ~10-fold increase in AST and ~5-fold increase in ALT, was observed in Ad-leptin, but not HD-leptin treated mice. Liver histopathology at these time points revealed evidence of inflammation and cellular infiltration in livers of Ad-leptin-treated mice, whereas livers from HD-leptin-treated mice were histologically indistinguishable from untreated control livers. These results indicated that HDAd were significantly less toxic than their FG counterparts. Importantly, in contrast to *ob*/*ob* mice, sustained high levels of serum leptin were observed in lean mice, for which leptin would not be a foreign antigen, following injection of HD-leptin but not Ad-leptin (62). Therefore, this study, together with the hAAT baboon study (79), suggested that in the absence of an immune response to the transgene product HDAds can provide prolonged transgene expression to achieve phenotypic correction of a genetic disease with negligible toxicity.

Kim et al. (66) investigated correction of hypercholesterolemia in apolipoprotein E (apoE)-deficient mice by using either an FGAd-encoding mouse apoE cDNA (FG-Ad5-cE), an HDAd-encoding mouse apoE cDNA (HD-Ad5-cE), or an HDAd-bearing mouse genomic apoE locus (HD-Ad5-gE). Intravenous injection of ApoE-deficient mice with 5×10^{12} particles/kg of FG-Ad5-cE resulted in an immediate fall in plasma cholesterol levels to within normal range (Fig. 6A). However, this effect was transient and plasma cholesterol levels increased after 28 days, returning to pretreatment levels by 112 days. Correlative with the plasma cholesterol levels, the levels of plasma apoE immediately increased shortly after injection but rapidly declined to pretreatment levels by day 28 (Fig. 6B). Similarly, intravenous injection of 7.5×10^{12} particles/kg of HD-Ad5-cE produced a complete and immediate lowering of plasma cholesterol to normal levels, but in contrast to FG-Ad5-cE, the reduced levels lasted about 1 year before gradually increasing (Fig. 6A). ApoE appeared in plasma within 1 week and remained at a level ~25% of wild-type (<10% of normal plasma levels of apoE is sufficient to maintain normal plasma cholesterol) but slowly declined to <10% of wild-type after about 1.5 years (Fig. 6B), at which time plasma cholesterol levels rose to ~50% of untreated mice (Fig. 6A). Intravenous injection of 7.5×10^{12} particles/kg of HD-Ad5-gE also resulted in a complete and immediate lowering of plasma cholesterol to subnormal levels for about 9 months, with levels subsequently staying within the normal range for the rest of the natural lifespan of the animal (2.5 years) (Fig. 6A). In this case, plasma apoE reached ~200% wild-type levels within 4 weeks and remained at supraphysiological levels for >4 months, at which time it slowly declined to about wild-type levels at 1 year and remained at 60% to 90% physiological concentrations for the lifetime of the animals (2.5 years) (Fig. 6B).

Because the duration of ApoE expression from HD-Ad5-gE was superior to HD-Ad5-cE, it would appear that genomic-based transgenes may be more effective than cDNA-based transgenes. Although the duration of expression from HD-Ad5-gE was impressive, lasting the lifetime of the mice, it would likely not be sustained in animals with significantly

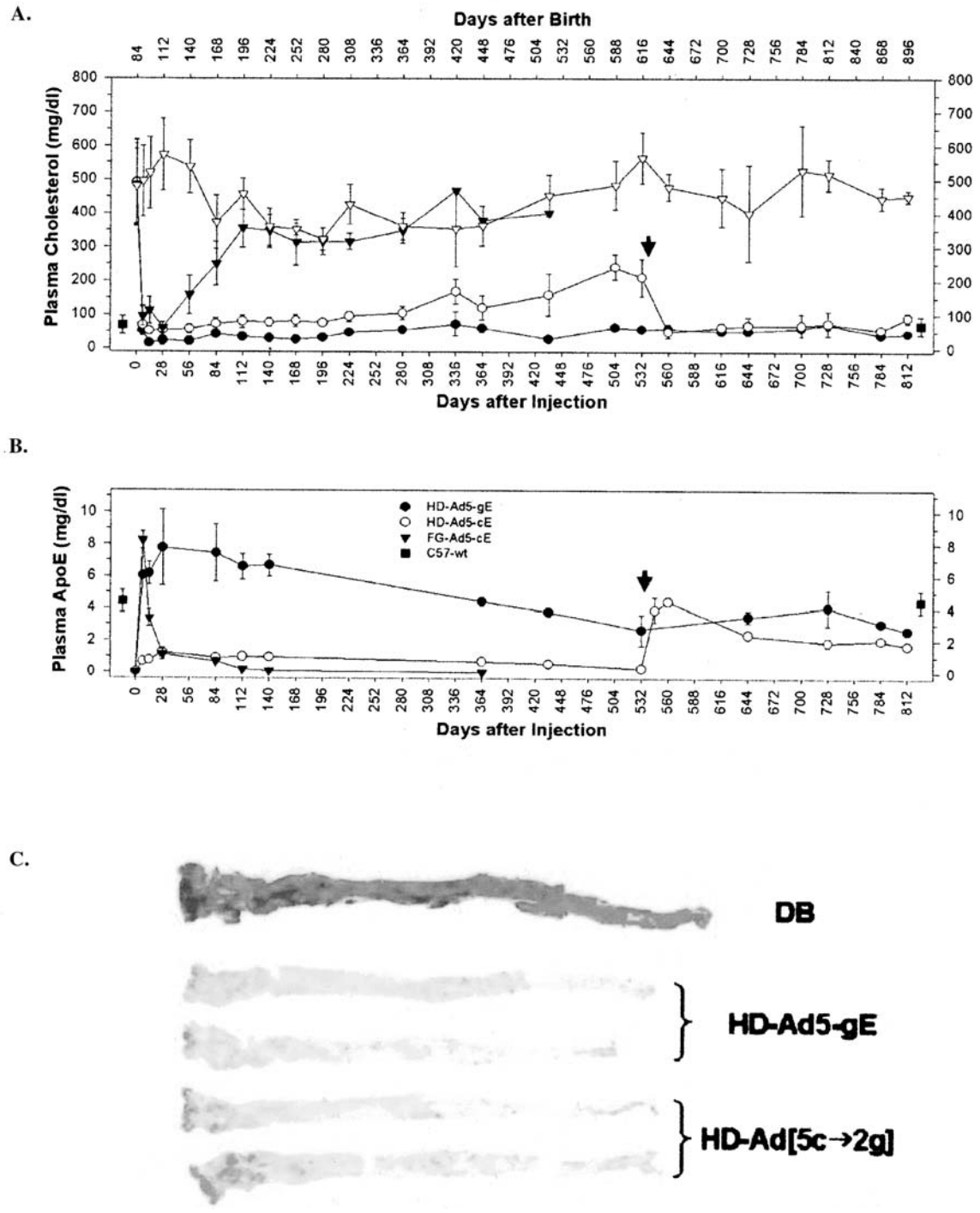

Figure 6 HDAd-mediated phenotypic correction of atherosclerosis in ApoE-deficient mice. (A) Plasma cholesterol and (B) plasma ApoE levels in ApoE-deficent mice injected with dialysis buffer (white triangle), FG-Ad5-cE (black triangle), HD-Ad5-gE (black circle), or HD-Ad5-cE followed (indicated by the bold arrow) by HD-Ad2-gE (white circle). (C) Aortas from HDAd-treated and control dialysis buffer (DB)-treated mice stained with Oil Red at 2.3 years postinjection. Atherosclerotic lesion areas, stained red, determined by quantitative morphometry were 91.45 mm^2 for DB-treated animals; 0.81 mm^2 and 0.31 mm^2 for HD-Ad5-gE-treated animals; and 5.89 mm^2 and 1.74 mm^2 for HD-Ad5-cE followed by HD-Ad2-gE-treated animals. See the color insert for a color version of this figure. (Modified from Ref. 66, copyright 2001 National Academy of Sciences, USA.)

longer lifespans, such as humans. Simply readministering the vector when transgene expression wanes is not possible due to the potent neutralizing anti-Ad antibody response that is elicited by the first administration. Indeed, mice previously treated with HD-Ad5-cE could not be successfully retreated again with the same vector. One solution to overcoming this problem is to administer a vector of a different serotype (72) (see Section V.F). To evaluate this strategy, Kim et al. generated a serotype 2 version of the genomic ApoE vector (HD-Ad2-gE) (66), using the serotype 2 helper virus described by Parks et al., (72) and showed that it could be successfully administered to mice previously treated with the serotype 5 HD-Ad5-cE to lower plasma cholesterol levels and raise plasma ApoE levels for the remainder of the animals' lives (Figs. 6A and 6B).

Aortas in all mice, examined at 2.3 years after treatment with HDAds, were essentially free of atherosclerotic lesions as determined by quantitative morphometry (Fig. 6C), demonstrating that a single injection of HDAd-encoding ApoE could confer lifetime protection against aortic atherosclerosis. Kim et al. also investigated the associated toxicities and found that, whereas injection of FG-Ad5-cE resulted in significant hepatotoxicity as indicated by significant elevation of AST and ALT (>10- to 20-fold), no such evidence of damage was observed following injection of any of the various HDAd constructs, even after a second administration with the serotype 2 HDAd (66).

In summary, this study elegantly demonstrated many of the advantages of HDAds for gene therapy. First, the large cloning capacity of the vector permits delivery of transgenes in their native chromosomal context, resulting in superior kinetics and duration of expression. Indeed, a single intravenous injection of HDAd, in this case, resulted in lifelong expression of the therapeutic transgene and permanent phenotypic correction of a genetic disease. Second, if transgene expression diminishes over time, administration of an alternative serotype HDAd is effective at circumventing the humoral immune response generated by the initial treatment. Third, negligible hepatotoxicity was associated with HDAd administrations.

Hemophilia A is the most common inherited severe bleeding disorder; it is caused by a deficiency in coagulation factor VIII (FVIII) and affects about 1 in 10,000 males. Patients with <1% normal plasma FVIII activity suffer from spontaneous and prolonged bleeding into joints, muscle, and internal organs. Hemophilia A is an attractive target for gene therapy because it is caused by a single gene defect, and even moderate increases of FVIII levels can convert a severe phenotype to a milder form. Reddy et al. (41) compared the efficacy and safety of hemophilia A gene therapy using a multiply deleted (E1, E2a, E3 deleted) Ad vector (Av3H8101) and an HDAd (AGV15huFVIII), both containing the identical B-domain-deleted human FVIII expression cassette. In this study, hemophiliac A mice were intravenously injected with 6×10^{10} particles of AGV15huFVIII or Av3H8101. Plasma hFVIII levels in mice treated with AGV15huFVIII peaked at 2 weeks postinjection and were 10-fold higher than levels achieved using Av3H8101 (Fig. 7A). Expression of hFVIII in AGV15-huFVIII-treated mice was sustained for at least 40 weeks although a ~10-fold decrease in plasma levels was observed between weeks 2 and 40 (Fig. 7A). In contrast, plasma hFVIII levels in Av3H8101-treated mice rapidly decreased to below the limit of detection (<25 mU/mL) by 12 weeks (Fig. 7A). It is interesting to note that the decrease in plasma hFVIII levels in both AGV15huFVIII and Av3H8101 mice was not due to the development of anti-hFVIII antibodies. At a dose of 3×10^{11} particles (1.5×10^{13} particles/kg), both AGV15-huFVIII and Av3H8101 induced hepatotoxicity as evident by ~10-fold increase in AST and ALT levels measured 1 day after vector administration (Fig. 7B). These levels returned to baseline by day 3. However, by day 7, animals treated with Av3H8101 showed a 10-fold elevation in AST and ALT levels, whereas those treated with AGV15huFVIII remained at baseline levels (Fig. 7B). AST and ALT levels did not return to baseline levels until day 28 in the Av3H8101-treated animals. These results suggested that the initial increase in liver transaminases observed at day 1 was caused by direct toxicity of the virion capsid protein from both AGV15huFVIII and Av3H8101. The toxicity observed at day 7 and beyond for Av3H8101, but not AGV15huFVIII, may have been due to viral gene expression from the Av3H8101 backbone. This study, unlike most others (see above), investigated vector-mediated toxicity shortly after administration and showed that HDAd can cause acute hepatoxicity by day 1. This toxicity was resolved by day 3, explaining why most others (who measured hepatotoxicity ≥3 days postinjection) have not observed hepatoxicity with HDAds.

Reddy et al. (41) also investigated the affect of helper virus contamination on the level and duration of hFVIII expression, vector-mediated toxicity, and development of anti-hFVIII antibodies, and found that these parameters were unaffected following intravenous injection of AGV15huFVIII preparations with 0.5%, 1%, 2.5%, 5%, and 10% helper virus contamination.

B. Transduction of Muscle

Duchenne muscular dystrophy (DMD) is a lethal, X-linked, degenerative muscle disease with a frequency of 1 in 3500 male births caused by mutations in the dystrophin gene. Dystrophin is an essential structural component of the skeletal muscle cell membrane, linking intracellular actin filaments with the dystrophin-associated proteins (DAPs) in the sarcolemma. Dystrophin deficiency results in instability of the muscle cell membrane causing muscle fiber degeneration. The length of the dystrophin cDNA (14 kb) precluded its inclusion into most gene therapy viral vectors. Following the development of HDAds with large cloning capacity, gene transfer of the full-length dystrophin cDNA became feasible. Indeed, the first in vivo application of HDAds was transduction of skeletal muscle for DMD gene therapy (50). In that study, an HDAd, AdDYSβgal, was constructed bearing the dystrophin cDNA under the control of a muscle-specific murine creatin kinase promoter and the LacZ reporter gene under the control of the CMV promoter (50). Direct intramuscular injection of 2×10^7 particles of AdDYSβgal into the gastrocnemius muscle

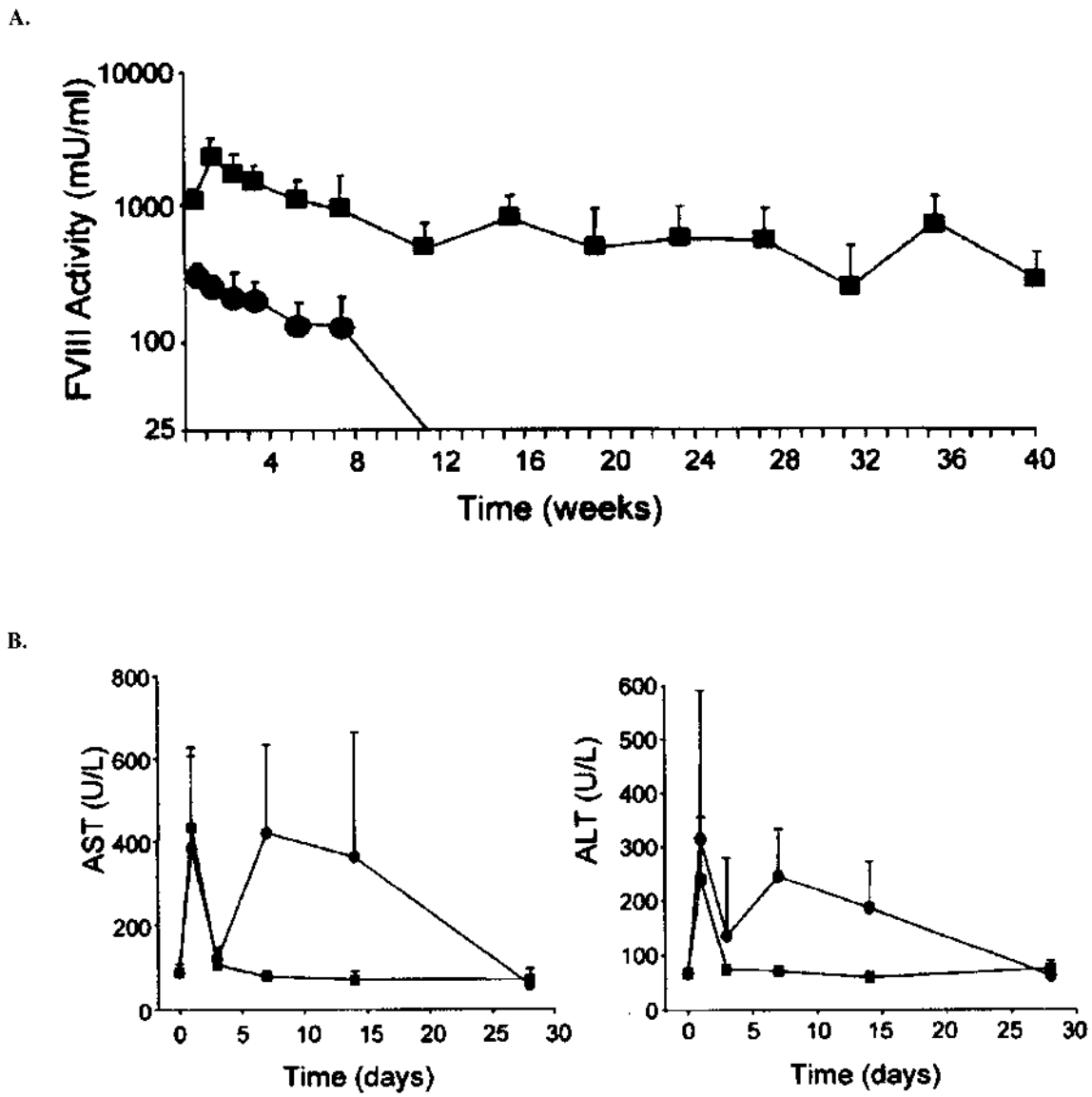

Figure 7 (A) Comparison of serum hFVIII levels in hemophiliac mice injected with 6×10^{10} particles of the HDAd AGV15huFVIII (squares) or the multiply deleted Ad Av3H8101 (circles). (B) Comparison of hepatotoxicity, as determined by measuring the serum levels of AST and ALT, in hemophiliac mice injected with 3×10^{11} particles of AGV15huFVIII (squares) or Av3H8101 (circles). (Modified from Ref. 41.)

of *mdx* mice, a genetic and biochemical model for DMD, resulted in expression of the 400-kDa full-length dystrophin protein, which was correctly localized to the sarcolemma membrane, restoring the DAPs to the muscle membrane and resulting in significant improvement of the histological phenotype (80). Most muscle fibers that expressed dystrophin also expressed β-galactosidase. However, by 6 weeks postinjection, the proportion of muscle fibers expressing dystrophin and β-galactosidase decreased. Expression of β-galactosidase from AdDYSβgal was identified as the principle cause of the loss of dystrophin expression (81). When immune response to β-galactosidase was eliminated, transgene expression persisted for at least 84 days with no significant loss of the AdDYSβgal vector DNA (81). Of course, for clinical use, therapeutic vectors would not contain an immunogenic reporter transgene such as LacZ.

More recently, Gilbert et al. (82) demonstrated that a single injection of an HDAd carrying two copies of the full-length human dystrophin cDNA under the control of a powerful hybrid CMV-enhancer/β-actin promoter, resulted in transduction of 34% of the fibers of the total tibialis anterior (TA) muscle in neonatal *mdx* mice. The amount of dystrophin produced in these muscles was 5 times that in normal human muscle, as determined by Western blot analyses. However, only 7% transduction was achieved following injection into the TA muscle of adult *mdx* mice. In these transduced adult fibers, the amount of dystrophin produced was only 10% of the amount in normal humans. However, the high levels of transduction were transient and a humoral immune response was mounted against the foreign human dystrophin protein in the *mdx* mice. Importantly, such a response was not observed in immunodeficient SCID mice suggesting that sustained expression could be achieved in the absence of an immune response to the transgene product. Interestingly, a 6-fold increase in transduction was observed in TA muscles by the HDAd in the presence of an FGAd vector. The authors proposed that gene products synthesized by the cotransducing FGAd acted in *trans* to increase transgene expression from the HDAd. The exact mechanism responsible for this intriguing result remains to be fully elucidated, although the Ad E4 region has been implicated to play a role (83).

Despite these encouraging results, effective gene therapy for DMD would likely require transduction of a critical number of muscle fibers in numerous major muscle groups throughout the body, including the limb, trunk, and respiratory muscles, a feat that is not currently feasible. However, muscle remains an attractive tissue for transduction because, like the liver, it may serve a more general purpose in gene therapy: (1) muscle constitutes as much as 40% of the total body mass and much of it is readily accessible, (2) skeletal myocytes can be transduced in vivo, (3) skeletal myofibers have a relatively long half-life and therefore represent a stable platform for transgene expression, (4) muscle is highly vascularized and skeletal muscle can efficiently secrete recombinant proteins into the circulation for systemic delivery, and (5) the high seroprevalence in the adult human population of preexisting anti-Ad neutralizing antibodies, an obstacle for intravenous vector delivery, may be minimized through localized delivery into the muscle. This strategy was investigated in a study by Maione et al. (84) in which mice were intramuscularly injected with C4AFO-mEPO, an HDAd-expressing mouse erythropoietin, as a marker gene. All mice intramuscularly injected with a dose of 1×10^6 infecious units (i.u.) or 3×10^5 i.u. retained transgene expression for at least 4 months. At a dose of 3×10^6 i.u., 30% of the mice slowly lost transgene expression. In contrast, all mice injected intramuscularly with FGAd lost transgene expression by day 21. The effect of preexisting anti-Ad antibody on the effectiveness of intramuscular administration was also investigated. Mice were first immunized with 1×10^{10} particles of a FGAd and, following generation of anti-Ad antibodies, the mice were challenged with intramuscular injections of HDAd at various doses. The authors found that successful intramuscular gene transfer could be accomplished in preimmunized mice, although a 30- to 100-fold higher dose was required to achieve 87% and 100%, respectively, the levels of transgene expression in naive mice. This is in sharp contrast to intravenous gene transfer in which 60% of preimmunized mice were completely refractory to transduction, whereas the remaining 40% were transduced but exhibited transgene expression levels comparable to naive mice intravenously injected with 1000-fold lower dose.

C. Transduction of the Brain

Stable, long-term transgene expression, in the absence of toxicity, is critical for the use of gene therapy to treat central nervous system (CNS) structural and functional deficits, such as aging-related memory loss, Parkinson's disease, Alzheimer's disease, or amyotrophic lateral sclerosis. Zou et al. (85) compared the efficiency, toxicity, and persistence of HDAd and FGAd vector-mediated gene transfer into the CNS of 20-month-old rats. An FG Ad vector or an HDAd-expressing β-galactosidase (5×10^8 particles) was stereotactically injected into the hippocampus or lateral ventricle. Transgene expression peaked 6 days postinjection for both vectors. In the hippocampus, transgene expression from the FGAd decreased rapidly after day 6, being significantly lower than HDAd-injected hippocampus by day 16, and undetectable by day 183. In the ventricle, transgene expression from FGAd was significantly lower than HDAd by day 33 and undetectable by day 66. In contrast, transgene expression from the HDAd remained relatively stable with expression remaining >60% of peak levels on day 183. Overall, expression from HDAd was significantly higher than from FGAd at all time points after 6 days. FGAd induced a substantial inflammatory and immune response as indicated by the presence, proliferation, and activation of microglia and asytocytes, as well as lymphocyte infiltration, which were significantly higher than those induced by HDAd at all time points after 3 h. Although both vectors induced a rapid increase in the proinflammatory cytokine IL-1β that peaked at 3 h postinjection, by 3 days, the level of IL-1β was significantly lower in the HDAd-treated brains than in the FGAd-treated brains. Similarly, both vectors induced the proinflammatory cytokine tumor necrosis factor (TNF)-α reaching peak levels by 3 to 6 h. However, the level of TNF-α remained significantly lower following injection of HDAd compared with FGAd. These results, along with those of Reddy et al. (41) (see Section VI.A) suggest that, although HDAd are significantly less toxic than FG and multiply deleted Ads, they are not completely benign.

VII. OUTSTANDING ISSUES

A. Large-scale Production

For those skilled in the art, the Cre/loxP system has been adequate for producing modest amounts of vector suitable for small animal experiments and limited low-dose large animal studies. However, the system's complexity, requiring a helper virus and multiple serial coinfections, renders large-scale vec-

tor production time consuming, laborious, and costly, especially for those with little practical experience with Ads. Although technical in nature, this difficulty is arguably the most significant obstacle currently hindering the progress of this promising gene therapy technology. Large quantities of vector will certainly be required for meaningful safety and efficacy studies of high-dose administration in large animal models and for clinical applications. Clearly, development of improved methods for large-scale vector production will be needed if this technology is to progress beyond the laboratory and into the clinic.

B. Helper Virus Contamination

HDAd produced using the current systems (54,55,59) are invariably contaminated with helper virus. Currently, the final level of helper virus contamination using these systems is $\leq$0.1% as determined by DNA-based methods of quantification (Southern blot hybridization or real-time PCR). Although these levels of contamination are low, they may not be acceptable for human use under conditions where high doses are needed. However, it should be noted that, even if high doses of HDAd were administered, a contamination of $\sim$0.1% would still represent a very small amount of helper virus (which is essentially an FGAd) relative to the much larger amounts of FGAd that have been given to numerous patients in clinical trials without adverse effect. It should also be noted that in mouse models, intravenous (41) or intramuscular (84) injection of HDAd with up to 10% helper virus contamination did not reduce the duration of transgene expression or result in significantly higher toxicity compared with preparations with only 0.1% to 0.5% contamination. However, because mice are much more tolerant to high doses of Ad, it remains to be determined whether this also holds true for larger animals.

C. Acute Toxicity

FG and multiply deleted Ads result in long-term, chronic toxicity owing, in large part, to expression of viral genes from the vector backbone in transduced cells rendering them targets for CTLs. A plethora of studies have now convincingly demonstrated that HDAds are considerably safer than FG and multiply deleted Ads in terms of long-term, chronic toxicity due to the absence of viral coding sequences. However, in addition to chronic toxicity, systemic high-dose administration of FG and multiply deleted Ads also results in acute toxicity in mice (86), nonhuman primates (87–89), and humans (90). Schnell et al. (88) proposed that Ad-mediated acute toxicity results from activation of the innate immune response as a consequence of Ad vector uptake by antigen-presenting cells (APCs). As a consequence, these APCs are immediately activated to secrete large amounts of inflammatory cytokines. This can lead to devastating consequences, which in its most severe form is lethal systemic inflammatory response syndrome (SIRS). For nonhuman primates, systemic injection of a FGAd at $\geq 1 \times 10^{13}$ vp/kg is lethal (87,89), whereas a dose of 6×10^{11} vp/kg of a multiply deleted (E1 and E4 deleted) Ad vector was lethal in a partially OTC-deficient patient who developed SIRS and succumbed to adult respiratory distress syndrome and multiorgan failure (88,90). Ad-mediated innate immune response occurs within hours of virus administration, and is believed to be unrelated to expression of viral genes from the vector backbone, but rather triggered by the Ad capsid proteins. This model would imply that HDAd, like FGAd, would also elicit a potent innate immune activation following high-dose systemic delivery into humans and nonhuman primates, with possible devastating consequences. Indeed, this notion is further supported by the observation that high-dose systemic injection into nonhuman primates of an FGAd rendered transcriptionally inactive by exposure to psorlen and ultraviolet irradiation, but still able to bind and enter cells, yielded similar laboratory and clinical manifestations of innate immune activation as did biologically active FGAd (88). It is important to note that while activation of innate immunity is also observed in mice given comparable systemic doses of biologically active or inactivated FGAd, lethal SIRS does not occur and may reflect species-to-species differences in the quality of the innate immune response or sensitivities of the end organs to pathological sequelae (91). Thus, it is important to ascertain whether HDAd would also elicit an acute, innate immune response as early generation Ad vectors do in nonhuman primates, and if so, to determine whether there are any qualitative and quantitative differences in the responses. Unfortunately, the challenge of producing the large quantities of HDAd required for such experiments remains a significant obstacle that first must be addressed (see above).

D. Immune Response to the Transgene Product

The most encouraging results demonstrating prolonged transgene expression from HDAd were all obtained in cases where an immune response was not mounted against the transgene product (62,66). However, in cases where an antibody response was mounted against the transgene product, expression was shortened, although to a lesser extent than when FGAds were used (62). Clearly immune response to the therapeutic proteins is an important issue because they may be antigenic in individuals suffering from some genetic deficiencies. This situation is, of course, not unique to HDAds but universal to gene therapy, regardless of vector type employed. Clearly, strategies such as immunosuppression or induction of tolerance to circumvent this obstacle may be essential for successful gene therapy of many genetic diseases.

VIII. CONCLUDING REMARKS

HDAds offer a number of advantages as gene transfer vehicles for gene therapy. The absence of viral coding sequences results in significant reduction in chronic toxicity and prolongation of transgene expression. Their large cloning capacity permits insertion of large genes, inclusion of *cis*-acting regulatory elements for regulated transgene expression and, in some

cases, insertion of genes in their genomic context for endogenous regulation, none of which may be possible with other viral vectors. The HDAd genome does not integrate into the host cell chromosomes but probably remains episomal within the nucleus. Although this precludes permanency and may be a serious limitation for transduction of rapidly dividing cell populations, it also minimizes the possibility of germline transmission or insertional mutagenesis, which may lead to oncogenic transformation, as may be possible with integrating vectors such as those based on retroviruses (92) or lentiviruses. Several important issues remain to be addressed before the full potential of this technology can be realized. Chief among these are improved methods for large-scale production, evaluation of acute toxicity following high-dose systemic delivery in a large animals, and the problem of host immune response to the therapeutic protein.

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5

Adeno-associated Virus and AAV Vectors for Gene Delivery

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I. INTRODUCTION

In this chapter we summarize the state of development of adeno-associated virus (AAV) vectors and provide an overview of AAV as well as some historical comment on early seminal studies that are generally overlooked in current reviews. We do not attempt to provide an exhaustive collection of references on development of AAV vectors but do discuss the key advances in the last several years, including improvements in vector production and studies on the applications for persistent gene expression. Other references provide general reviews of AAV (1,2) and extensive summaries on earlier development of AAV vectors (3–7).

AAV vectors have a number of advantageous properties as gene delivery vehicles. The parental virus does not cause disease. AAV vectors are the smallest and most chemically defined particulate gene delivery system and potentially could be classified as well-characterized biologics for therapeutic applications. AAV vectors contain no viral genes that could elicit undesirable cellular immune responses and appear not to induce inflammatory responses. The primary host response that might impact use of AAV vectors is a neutralizing antibody response. The vectors readily transduce dividing or nondividing cells and can persist essentially for the lifetime of the cell. Thus, AAV vectors can mediate impressive long-term gene expression when administered in vivo. Consequently, these vectors may be well suited for applications where the vector is delivered infrequently and where any potential host antibody response to the AAV capsid protein may be less inhibitory. One limitation for AAV vectors is the limited DNA payload capacity of about 4.5 kilobases (kb) per particle.

The lack of good production systems that could generate high titer vectors was an early obstacle to development of AAV vectors, but this has been overcome through significant advances in both upstream production and downstream purification of AAV vectors. Clinical development of AAV vectors has progressed significantly and studies of an AAV vector in cystic fibrosis patients (8–11) have been extended to phase II trials. Other AAV vectors have now entered clinical trials for hemophilia B (12) and limb girdle muscular dystrophy (13).

Since the first edition of this book (14), there have been extensive advances in application of AAV vectors in many animal models and further analysis of AAV vector safety profiles and host cell responses. There have also been remarkable advances in understanding the structure and biology of AAV vectors, including uptake into cells, trafficking to the cell nucleus, and the mechanism of genome persistence. These studies suggest possible ways to modify the biological targeting of AAV vectors, enhance transduction efficiency, and overcome the packaging limitation.

Most of the early studies on AAV used AAV serotype 2, but genomes of several other AAV serotypes have been cloned and sequenced (15). The biological properties of individual serotypes include differences in the interactions with cellular receptors (16). Other studies are now providing information on the structure of the AAV capsid and how its interaction with the cell may be modified. Notably, the crystal structure of AAV2 was recently described (17). Thus, together with additional studies on cellular trafficking pathways, it may be possible to modify the targeting of AAV vectors as well as to enhance their transduction efficiency (18).

Studies on the mechanism of persistence of vector genomes in transduced cells indicate that this involves formation of

polymeric DNA structures or concatemers. Concatemers can also be formed between 2 different vector genomes introduced into the same cell. This provides a way to partly circumvent the packaging limit of AAV by dividing a gene expression cassette between 2 AAV vectors (''dual vectors'') and allowing recombination in the cell to generate the intact expression cassette.

II. ADENO-ASSOCIATED VIRUS

A. AAV Discovery

AAV is a small, DNA-containing virus that belongs to the family Parvoviridae within the genus *Dependovirus*. AAV was originally observed as a contaminant of laboratory preparations of adenovirus, then was recognized as a virus that was different from adenovirus but that was dependent on adenovirus for its replication (19,20). Soon after the discovery of AAV in laboratory stocks of adenoviruses, it was isolated from humans (21). AAV has not been associated with any disease but has been isolated from humans, generally in association with an infection by adenovirus (21).

Several serotypes of AAV have been distinguished. AAV1, AAV2, AAV3, and AAV4 have extensive DNA homology and significant serological overlap (22–25), but AAV5 is somewhat less related (26,27). AAV2 and AAV3 are the most frequently isolated serotypes from humans (21), whereas AAV5 has been isolated from humans only once (28). AAV4 is a simian isolate that does not infect humans, and AAV1 originally may have been isolated from a simian source (29). Additional recent isolates, AAV6 (30), AAV 7, and AAV8 (31), are discussed below. Other AAV have been identified in a variety of animal species, but little characterization of these AAV isolates has been reported (1).

B. Epidemiology

In the United States, a significant proportion of the population over age 10 years may be seropositive for AAV2 and AAV3 (21). AAV2 and AAV3 appear to be transmitted primarily in nursery populations in conjunction with the helper adenovirus and thus also appear to be replication defective in the natural human host. A signal epidemiological study of AAV was carried out in a population of children in an orphanage in Washington, DC, in whom seroconversion to AAV was observed during the course of an adenovirus infection (21). In infected individuals, the virus is shed in body fluids including sputum and stool. It is noteworthy that the early epidemiological studies also analyzed neutralizing antibody responses to AAV in humans. The presence of serum-neutralizing antibody against AAV2 or AAV3 did not prevent reinfection of humans but did prevent shedding of the virus (21). This observation is significant for use of AAV as a gene delivery vector because it suggests that repeat delivery may be feasible (32).

C. Biology of AAV Life Cycle

AAV is a defective parvovirus that replicates only in cells in which certain functions are provided by a coinfecting helper virus, generally an adenovirus or a herpes-virus (1). AAV has both a broad host range and wide cell and tissue specificity, and replicates in many cell lines of human, simian, or rodent origin provided an appropriate helper virus is present. There may be some limitations to AAV tissue specificity in vivo or at least some significant differences in efficiency of transduction of different tissues and organs. These limitations may reflect the receptor and coreceptors apparently used by different AAV serotypes for entry into cells as well as cellular trafficking of AAV. This aspect of AAV biology is becoming of increasing importance for development of AAV vectors. A second set of parameters that may impact AAV tissue and organ specificity and its replication reflect the nature of the helper function provided by helper viruses.

An additional event required by AAV to function efficiently as a gene delivery vehicle is the need to convert the incoming single-stranded DNA genome to a double-stranded molecule to permit transcription and gene expression. This process is termed single-strand (ss) conversion or metabolic activation (33), and the rate at which it occurs may depend in part on the physiological state of the host cell. However, the process may be accelerated by treatment of the cell with genotoxic agents or by certain helper virus functions.

Infection of certain cell lines by AAV in the absence of helper functions results in its persistence as a latent provirus integrated into the host cell genome (34,35). In such cell lines, the integrated AAV genome may be rescued and replicated to yield a burst of infectious progeny AAV particles if the cells are superinfected with a helper virus such as adenovirus. Importantly, in cultured cells, AAV exhibits a high preference for integration at a specific region, the AAVS1 site, on human chromosome 19 (36,37). The efficiency and specificity of this process is mediated by the AAV *rep* gene (38–40). *Rep*-deleted AAV vectors do not retain specificity for integration into this chromosome 19 region (41) and indeed may not integrate efficiently but remain as episomes.

D. Mode of Cell Entry and Host Tropism

AAV appears to have a broad host range and different AAV serotypes replicate in vitro in many human cells and in a variety of simian and rodent cell lines if a helper virus with the appropriate host range is also present. AAV also infects various animal species, and human isolates of AAV will grow in mice or monkeys if the appropriate mouse or monkey adenovirus is also present. This indicated that cellular receptors for AAV were likely to be relatively common on many cell types.

Recent experiments demonstrated (42) that AAV2 particles can use heparin sulfate proteoglycans (HSPGs) as a receptor, and some cell lines that do not produce HSPGs are impaired for AAV binding and infection. Additional studies suggest that AAV2 also uses a coreceptor for efficient internalization and 2 possible coreceptors, $\alpha_v\beta_5$ integrin (43) and human fibroblast growth factor receptor 1 (FGFR1) (44), were identified.

It is of interest that the $\alpha_v\beta_5$ integrin coreceptor, which is used for a similar purpose by adenovirus type 2 and 5, is preferentially located on airway epithelial cells in the more distal areas of the conducting airway (45). This maybe important for use of an AAV2 gene therapy vector for cystic fibrosis because the distal airway is the region of the lung most impacted by the disease. It is also noteworthy that FGFR is expressed in most tissues but is of highest abundance in skeletal muscle and neuroblasts and glioblasts in the brain, and these 2 organs appear to be good targets for AAV2 transduction.

The existence of more than one coreceptor suggests that AAV may have multiple mechanisms for cell entry, and there is already some evidence to support this concept (46). Also, real-time imaging of entry into HeLa cells by individual AAV2 particles labeled with the dye Cy5 (47) showed that endocytosis was rapid and that some particles could reach the nucleus within 15 min of first contacting the cell. However, there was evidence of free diffusion of both endosomes and AAV particles, and also evidence for movement of each of these entities being driven by cellular motor proteins. Furthermore, cellular trafficking events following endocytosis of AAV that may involve the ubiquitin-proteosome pathway appear to play a significant role (48–50), as discussed in Section VIII.A.

Cell entry may also be impacted by the route of delivery, and AAV vectors can transduce airway cells when delivered directly to the lung (51,52), or brain cells (53) and myocytes (54,55) when delivered directly to these organs. However, when delivered intravenously by tail vein injection in mice (56,57), the vector preferentially accumulated in the liver, and this may reflect both the presence of a much more porous vasculature in the liver and also the small size of the AAV particles. The small size of the AAV particle may also be of advantage in passing through the basal lamina pores in muscle, thus accessing a large number of myoblasts and myotubes.

III. AAV MOLECULAR BIOLOGY

A. Particle Structure

AAV is a nonenveloped particle about 20 nm in diameter with icosahedral symmetry, which is stable to heat, mild proteolytic digestion, and nonionic detergents. The AAV particle is comprised of a protein coat, containing the 3 capsid proteins, VP1, VP2, and VP3, which encloses a linear single-stranded DNA genome having a molecular weight (mw) of 1.5×10^6. The VP1, VP2, and VP3 proteins are present in the viral capsid in the ratio of 1:1:8. The DNA represents 25% by mass of the particle that therefore exhibits a high buoyant density (1.41 g/cm^3) in cesium chloride. The relative stability of the AAV particle is an important property because it can withstand robust purification procedures, which facilitates scaled-up production of AAV vectors.

The crystal structure of AAV 2, determined at 3.0 Å resolution by X-ray crystallography, reveals several interesting features that will be helpful for efforts aimed at modifying the AAV capsid to alter targeting specificity (17). The structure shows that each capsid comprises 60 protein subunits arranged in T = 1 icosahedral symmetry. All the amino acids, except the 14 amino-terminal residues of VP1, could be localized in the structure. The surface of the capsid shows a distinctive topology with 3 peaks clustering around each 3-fold icosahedral axis (Fig. 1). Each 3-fold proximal peak is formed from 2 interacting protein subunits, and the sides of these peaks appear to be the regions that mediate the receptor-binding interactions with heparin sulfate. An additional feature of the AAV capsid is that, like all other parvoviruses, it appears to contain a phospholipase A2 activity that in AAV is located in the unique amino-terminal region of the VP1 protein (58), and this activity appears to be essential for infectivity to mediate exit from endosomes.

A novel feature of AAV is that, although each particle contains only one single-stranded genome, strands of either complementary sense, ''plus'' or ''minus'' strands, are packaged into individual particles. Equal numbers of AAV particles contain either a plus or minus strand. Either strand is equally infectious and AAV displays single-hit kinetics for infectivity (59).

When DNA is extracted from AAV particles, the plus and minus strands anneal to generate duplex molecules of 3.0 ×

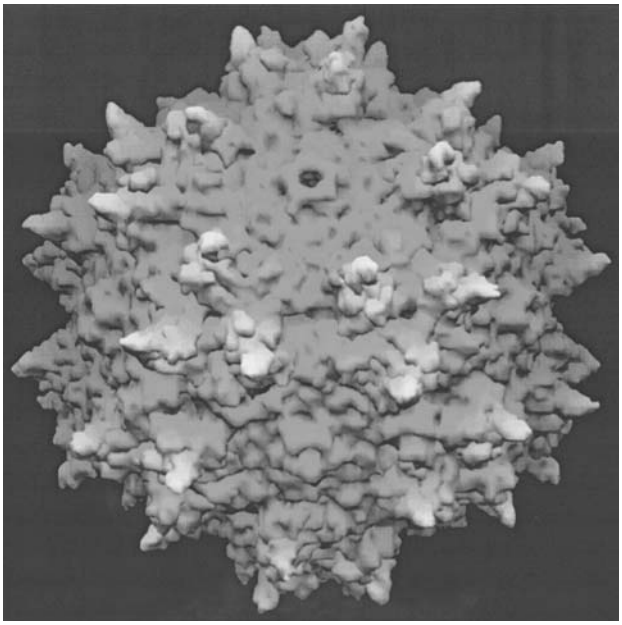

Figure 1 Structure of AAV serotype 2. The structure was determined by X-ray crystallography at a resolution of 3.0 Å. The surface topology is shown drawn to scale. The view is down a 2-fold axis (center of the virus) with 3 folds left and right of center, and 5 folds above and below. Overall, the outside surface is positively charged with a prominent ring of symmetry-related positive patches in a depression surrounding the 5-fold axis. See the color insert for a color version of this figure. (Reprinted with permission from Ref. 17.)

10^6 mw. However, Crawford and his colleagues (60) showed that, on the basis of a careful physical characterization of AAV particles, each particle appeared to contain only DNA of 1.5 $\times$ 10^6 mw. They suggested that the only way to reconcile this conundrum was to propose that individual plus and minus strands must be packaged into individual particles. An elegant proof of this conundrum was provided by Rose and his colleagues (61) who made 2 preparations of AAV particles, in which one preparation had thymidine substituted by bromodeoxyuridine (BudR) and the other was unsubstituted. The preparations of particles were mixed prior to extraction of DNA. Analysis of the duplex DNA obtained upon extraction showed components with intermediate density formed by individual strands from substituted or unsubstituted particles that had annealed during extraction. This constituted formal proof of the novel DNA strand segregation exhibited by AAV during packaging of its DNA. BudR substitution of AAV DNA also permits separation of the plus and minus strands, and this was used along with 5′ end-labeling and restriction endonuclease cleavage to determine the strand polarity of the AAV genome (62).

B. AAV Genome Structure

The AAV2 DNA genome (22,63) is 4681 nucleotides long, and includes 1 copy of the 145-nucleotide long inverted terminal repeat (ITR) at each end and a unique sequence region of 4391 nucleotides long that contains 2 main open reading frames for the *rep* and *cap* genes (Fig. 2). The unique region contains 3 transcription promoters, p_5, p_{19}, and p_{40}, which are used to express the *rep* and *cap* genes. The ITR sequences are required in *cis* to provide functional origins of replication (*ori*), as well as signals for encapsidation, integration into the cell genome, and rescue from either host cell chromosomes or recombinant plasmids. The genomes of other AAV serotypes have been sequenced and appear to have a structure similar to AAV2 (24–26,30).

The *rep* gene is transcribed from 2 promoters, p_5 and p_{19}, to generate 2 families of transcripts and 2 families of rep proteins (Fig. 1). In addition, splicing of these mRNAs yields 2 different carboxyl terminal regions in the rep proteins. The capsid gene is expressed from transcripts from the p_{40} promoter that accumulate as two 2.3-kb mRNAs that are alternately spliced. The majority 2.3-kb transcript codes for the VP3 protein initiated from a consensus AUG initiation codon. However, at about a 10-fold lower frequency, translation of this transcript also occurs slightly upstream at a nonconsensus ACG initiation codon to yield VP2. The minority 2.3-kb mRNA is spliced to an alternate 3′ donor site 30 nucleotides upstream, and this retains an AUG codon that is used to initiate translation of VP1. Thus, VP1 and VP2 have the same polypeptide sequence as VP3 but have additional amino-terminal sequences. This elegant arrangement results in generation of VP1, VP2, and VP3 in ratios of about 1:1:8, which is the same as the ratio of these proteins in the viral particle.

C. Replication

In a productive infection in the presence of a helper such as adenovirus (4,64), the infecting parental AAV ss genome is converted to a parental duplex replicating form (RF) by a self-priming mechanism that takes advantage of the ability of the ITR to form a hairpin structure (Fig. 3). The parental RF molecule is then amplified to form a large pool of progeny RF molecules in a process that requires both the helper functions and the AAV *rep* gene products, Rep78 and Rep68. AAV RF genomes are a mixture of head-to-head or tail-to-tail multimers or concatemers and are precursors to progeny ss DNA genomes, which are packaged into preformed empty AAV capsids (65). Rep52 and Rep40 interact with the preformed capsid, apparently to provide a DNA helicase function for DNA packaging (66)

The kinetics of AAV replication and assembly has been investigated (64,67). In human HeLa or 293 cells simultaneously infected with AAV and adenovirus, there are 3 phases of the growth cycle. In the first 8 to 10 h, the cell becomes permissive for AAV replication as a result of expression of a subset of adenovirus genes, including E1, E2A, E4, and the VA RNA (68). During this period, the infecting AAV genome is converted to the initial parental duplex RF DNA by self-priming from the terminal base-paired 3′ hydroxyl group provided by the ability of the ITR to form a self-paired hairpin. This initial generation of a duplex genome also provides a template for transcription and expression of AAV proteins. In a second phase, from about 10 to 20 h after infection, the bulk of the AAV rep and cap proteins are synthesized, and there is a large amplification of monomeric and concatemeric duplex AAV RF genomes to a constant level (67). During the third phase of AAV growth, between 16 and 30 h, ss progeny molecules are synthesized by a strand-displacement replication mechanism and packaged into preformed capsids followed by accumulation of mature, infectious AAV particles (65,67).

The rep proteins perform important biochemical functions (4). Rep68 and Rep78 bind to the ITR and are site- and strand-specific endonucleases that cleave the hairpin in an RF molecule at the site that is the 5′ terminus of the mature strand. In addition, these proteins contain an ATP-binding site that is important for the enzymatic activity but not for binding to the ITR. Further, Rep78 and Rep68 have both DNA and RNA helicase activity. These rep proteins also regulate transcription (4,6,7). Rep78 is a negative autoregulator of the p5 promoter (i.e., of its own synthesis), but is an activator of the p40 promoter to enhance capsid protein production. Rep52 and Rep40 do not bind to the ITR but provide a helicase in assembly of mature particles. Also, the smaller rep proteins are antirepressors and block the negative autoregulation of p5 by Rep78 (69).

The AAV replication cycle is highly coordinated with respect to expression of rep and cap proteins and the relationship between replication and assembly (67,69). Any vector production process that provides the rep and cap functions by complementation may decrease the efficiency of this highly regulated

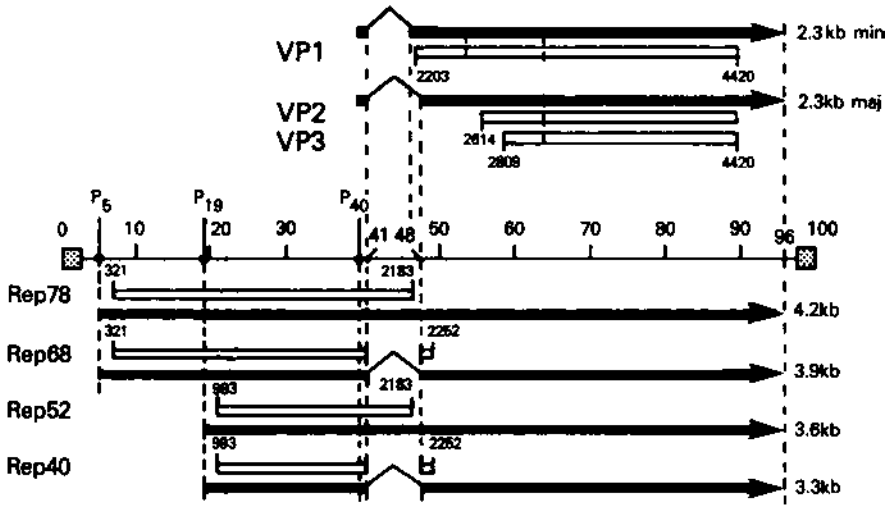

Figure 2 Structure of the AAV 2 genome. The AAV2 genome is shown as a single bar with a 100 map unit scale (1 map unit ≅ 47 nucleotides). Stippled boxes indicate inverted terminal repeats (ITRs; replication origins) and solid circles indicate transcription promoters (p5, p19, p40). The poly A site is at map position 96. RNAs from AAV promoters are shown as heavy arrows with introns indicated by the caret. The coding regions for the 4 rep proteins (Rep78, Rep68, Rep52, Rep40) and for the viral capsid proteins (VP1, VP2, VP3) are shown with open boxes, and the numbers indicate the locations of initiation and termination codons. (Reprinted with permission from Ref. 63.)

process. Nonetheless, AAV has one of the largest burst sizes of any virus and following infection of cells with AAV and adenovirus as helper, this may be well in excess of 100,000 particles per cell (11). These considerations imply that a high yield of AAV vector particles per cell theoretically is attainable. Attaining high specific productivity is of crucial importance in developing scaled-up vector production because the ability to obtain maximum yields ideally requires high specific productivity (yield of particles per cell) or large biomass (total number of cells). Maximizing the specific productivity may avoid unnecessary increases in biomass.

D. Genetics

The cloning of infectious AAV genomes in bacterial plasmids facilitated a molecular genetic analysis of AAV (70,71). These studies showed that the *rep* and *cap* genes are required in *trans* to provide functions for replication and encapsidation of viral genomes, respectively, and that the ITR is required in *cis* (4,6,63). Mutations in the ITR have an Ori phenotype and cannot be complemented in *trans*.

Mutations that affect the Rep78 and Rep68 proteins have a Rep phenotype, and are deficient for both the bulk replication and amplification of duplex RF molecules and for accumulation of ss, progeny genomes. A mutation that affected only the Rep52 and Rep40 proteins showed an Ssd phenotype in which duplex RF replication occurred normally but no ss progeny DNA accumulated.

The *cap* gene encodes the proteins VP1, VP2, and VP3 that share a common overlapping sequence, but VP1 and VP2 contain additional amino terminal sequence. All 3 proteins are required for capsid production. Mutations that affect VP2 or VP3 have a Cap phenotype, and block capsid assembly and prevent any accumulation of ss DNA. This indicates that VP3 and VP2 are primarily responsible for forming the capsid and that ss DNA does not accumulate unless it can be packaged into capsids. Mutations that affect only the amino terminus of the VP1 protein do not prevent accumulation of capsids or ss DNA, but no infectious AAV particles accumulate. This phenotype has been described as either Inf or Lip (low infectivity particles).

These genetic studies, together with additional biochemical studies, show that Rep68 and Rep78 are required for replication, that VP2 and VP3 are required to form the capsid, and that Rep52 and Rep40 appear to act in concert with VP1 to encapsidate the DNA and stabilize the particles (4,5,66). An additional role of VP1 appears to be to provide the phospholipase activity that is required for infectivity (58).

The rep proteins exhibit several pleiotropic regulatory activities, including positive and negative regulation of AAV genes and expression from some other viral or cellular promoters, as well as inhibitory effects on the host cell. Because of the inhibitory effects of expression of *rep* gene products on cell growth, expression of rep proteins in stable cell lines was difficult to achieve, and this delayed development of AAV packaging cell lines (72). For this reason, various approaches to AAV vector production have employed transient transfection of cells with AAV vector plasmids and complementing *rep-cap* plasmids. However, even in these transfection systems, the closely coordinated regulation of *rep* and *cap* gene

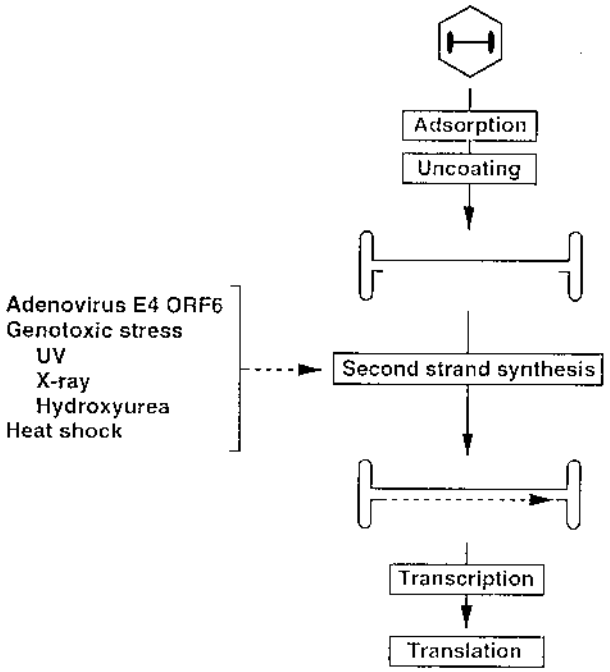

Figure 3 Metabolic pathway of AAV genomes in cells. Binding of AAV to cells is independent of helper virus functions. Trafficking of AAV to the nucleus may be enhanced by agents that interact with the ubiquitin pathway and proteosome processing. Conversion of the infecting single-strand genome to a duplex structure (or parental RF) through the process of metabolic activation (second-strand synthesis) can occur independently of helper virus. This process may be enhanced by infection with helper adenovirus genes such as E4orf6 or by other metabolic insults, including genotoxic stress or heat shock. Treatments that enhance metabolic activation may enhance gene expression from the vector template. The single strand and duplex strands are drawn to show the ITR in the base-paired hairpin conformation that allows self-priming of replication to form a duplex template using cellular DNA polymerases. For further details see text. (Reprinted with permission from Ref. 165.)

expression and the interactions of the 3 AAV promoters (69) are important considerations in optimizing vector production.

IV. AAV LATENCY AND PERSISTENCE

A. Latency

A U.S. government screening program to assess human cell lines for vaccine production led to the observation that infection of primary cultures of human embryonic kidney cells with adenovirus resulted in rescue of infectious AAV. The hypothesis that some cultures may have carried a latent form of AAV was tested directly (34) by infecting a human cell line, Detroit 6, with AAV at a high multiplicity of infection and passaging the cell cultures until no infectious AAV genomes were present, which required at least 10 cell passages. Following this, superinfection of the cultures with adenovirus resulted in rescue of infectious AAV. This provided an important demonstration of a way in which AAV may survive in a cell if conditions are not permissive for replication.

B. Integration

Analysis of a human cell line, Detroit 6, carrying latent AAV showed that the cells contained a relatively low number of AAV genomes that were integrated into the host cell chromosome, mostly as tandem repeats. Early studies of cell lines stably transduced with AAV vectors expressing selectable markers also showed that most stable copies in the cell existed as tandem repeats with a head-to-tail conformation (35). Analysis of chromosomal flanking sequences showed that, for wild-type AAV, 50% to 70% of these integration events occurred in a defined region (36,37). When wild-type AAV infects human cell lines in culture, up to 50% to 70% of these integration events occur at a region known as the AAVS1 site on chromosome 19 at 19q13ter. Both the specificity and efficiency of AAV integration are mediated by the AAV rep protein that binds to the ITR and to a site in the AAVS1 site on human chromosome 19 (38). These studies were performed on cells in culture, and it is noteworthy that naturally occurring, latent, integrated AAV genomes have not been well characterized in humans or any other animal species. In a single study, wt AAV infection of rhesus macaques was examined in the presence and absence of adenovirus infection (73). In this study, a wtAAV-AAVS1 host cell DNA junction could be detected by polymerase chain reaction (PCR) amplification in only 1 of 9 animals, suggesting that integration of AAV even under favorable conditions is very rare.

C. Vector Persistence

Some early studies with AAV vectors expressing selectable markers suggested that these vectors also integrated at AAVS1 (36,74). However, the vectors used in these studies were also were contaminated by wild-type AAV particles and may represent rare integration events mediated by rep and enriched by the selectable marker. In contrast, AAV vectors that contain no AAV *rep* coding sequences and no selectable marker have reduced efficiency and specificity for integration at the chromosome 19 AAVS1 site (40,41), and more usually persist in an episomal state. Initial evidence for this episomal persistence came from fluorescent in situ hybridization (FISH) analysis of cell lines transduced with AAV vectors, which carried a low number of copies of an AAV vector as determined by Southern blot hybridization. FISH analysis of interphase nuclei compared with FISH analysis of metaphase chromosomes showed a reduced proportion of the cells carrying all copies at a metaphase chromosomal site (41).

Studies performed in vivo in a variety of animal models now indicate that, in the absence of selective pressure, AAV

vectors generally persist as episomal genomes. A number of studies have now shown that AAV can persist for extended periods of time when administered in vivo (51–56) and that the predominant form of the persisting vector genomes appears to be multimeric structures, which are head-to-tail concatemers (52,75–78) that are circular (79–81). How these head-to-tail multimers are formed is unknown, but it cannot involve the normal AAV replication process because that requires rep protein and gives only head-to-head or tail-to-tail concatemers. Whether the circular concatemers are integration intermediates, as has been suggested for AAV integration (39,82), is also unknown. However, available evidence indicates that the majority of these head-to-tail concatemers are episomal and that integrated copies of vector in organs such as liver or muscle are very rare (83,84).

D. Targeted Integration

Although AAV vectors that do not contain the *rep* gene do not integrate at the AAVS1 site at any significant frequency, they can be directed to integrate at the chromosome 19 site by supplying the *rep* gene in *trans*. For instance, a plasmid containing an AAV vector comprised of a reporter gene between the AAV ITRs and having a rep gene also in the plasmid, but outside the ITRs, resulted in integration of the AAV ITR vector cassette into the chromosomal site at 19q in human 293 cells in vitro (85). Also, a baculovirus vector that contained an AAV reporter gene vector and separately contained an AAV rep gene was able to direct integration of the AAV vector into the AAVS1 site in human 293 cells (86). Similarly, cotransduction of an epithelial tumor cell line (HeLa) or a hepatoma cell line (HepG2) with 2 hybrid Ad/AAV viruses, one carrying an AAV vector and the other expressing the *rep* gene, resulted in integration of the AAV vector into the AAVS1 site (87).

In another study, the ability of AAV vectors to carry sequences that could mediate homologous recombination with chromosomal sequences was examined in cultured human cell lines. Homologous recombination at specific chromosomal sites could be attained (74), and additional studies showed that careful design of the interacting homologous sequences could raise the efficiency of the process to about 1% (88). However, this still requires a selective marker so its application to gene therapy may be limited.

V. AAV PERMISSIVITY

A. Helper Functions Provided by Other Viruses

The precise mechanism of the helper function provided by adenovirus or other helper viruses has not been clearly defined. These helper functions may be complex but relatively indirect and probably affect cellular physiology rather than providing viral proteins with specific functions in the AAV replication system. Studies with adenovirus (68) have clearly defined that only a limited set of adenovirus genes are required, and these comprise the early genes E1A, E1B, E2A, E4orf6, and the VA RNA. The primary role of E1A is to transcriptionally activate the other adenovirus genes, but it may also transcriptionally activate the AAV p5 promoter. E1B and the E4orf 6 protein of adenovirus interact to form a complex, and these 2 genes provide the minimal function required to permit AAV DNA replication. The E2A gene of adenovirus has a complex function because it is a ss DNA-binding protein that is directly involved in adenovirus replication; however, it also has an important role in regulating adenovirus gene expression. The role of E2A for AAV appears not to be a DNA replication function but to involve enhancement of AAV gene expression, particularly expression of AAV capsid protein. The VA RNA is also important in maximizing the level of AAV gene expression.

B. Alternate Pathways to Permissivity

The concept that the helper virus renders the cell permissive by enhancing AAV replication in an indirect way is consistent with the evidence that helper virus genes do not appear to provide enzymatic functions required for AAV DNA replication, and that these functions are provided by AAV rep protein and the cellular DNA replication apparatus (68). This is also consistent with the observations that in certain cell lines, particularly if they are transformed with an oncogene, helper-independent replication of AAV DNA can occur if the cells are also treated with genotoxic agents such as ultraviolet (UV) or x-irradiation, or with hydroxyurea (89,90). In these circumstances, a small proportion of the cells could be rendered permissive for AAV replication, but the level of replication and production of infectious AAV was very low.

C. Replication or Persistence

Two distinguishable phases of the AAV life cycle occur in permissive or nonpermissive cells. In either case, the infecting single-stranded genome is converted to a duplex structure. There is evidence that the single-stranded genome may be converted to either linear duplexes or circular duplex molecules (79,80). However, in permissive cells in the presence of helper virus, this duplex genome then appears to follow the pathway of bulk replication using the self-priming property of the ITR to yield a large pool of head-to-head and tail-to-tail RF molecules, and ultimately a large burst of progeny particles. In nonpermissive cells in the absence of helper, these genomes follow a pathway that leads to generation of head-to-tail concatemers that persist as episomes or become integrated into the host cell chromosome. In this nonpermissive state, there are 2 important parameters that may have different consequences for AAV or AAV vectors. In either case, there are no helper functions provided by another virus. However, for wild-type AAV, the *rep* gene is present and therefore may be expressed. This may explain why AAV integrates efficiently into the chromosome 19. For an AAV vector, the *rep* gene is not present; thus, vectors may progress through the integration

pathway more slowly, or not at all, and rather follow the pathway of persistence as a circular episome (83,84).

VI. AAV VECTORS

A. Design of AAV Vectors

The ability to generate AAV vectors was facilitated by the observation that molecular cloning of double-strand (ds) AAV DNA into bacterial plasmids followed by transfection into helper virus-infected mammalian cells resulted in rescue and replication of the AAV genome free of any plasmid sequence to yield a burst of infectious AAV particles (5). This rescue may occur by a mechanism analogous to that used in reactivation of a latent provirus after superinfection of cells with adenovirus. The general principles of AAV vector construction (4,5,12) are based on modifying the molecular clones by substituting the AAV coding sequence with foreign DNA to generate a vector plasmid. In the vector, only the *cis*-acting ITR sequences must be retained. The vector plasmid is introduced into producer cells that are also rendered permissive by an appropriate helper virus such as adenovirus. To achieve replication and encapsidation of the vector genome into AAV particles, the vector plasmid must be complemented for the *trans*-acting AAV *rep* and *cap* functions that were deleted in construction of the vector plasmid. AAV vector particles can be purified and concentrated from lysates of such producer cells.

The AAV capsid has 3 important effects for AAV vectors. There is a limit of about 5 kb of DNA that can be packaged in an AAV vector particle. This places constraints on inclusion of very large cDNAs and may also limit the ability to include extensive regulatory control sequences in the vector. The capsid also interacts with the AAV receptor and coreceptors on host cells, and thus mediates cell entry. The capsid may also induce humoral immune responses that could limit delivery of AAV vectors for some applications.

Except for the limitation on packaging size and the requirements for ITRs, there are no obvious limitations on the design of gene cassettes in AAV vectors. The ITR can function as a transcription promoter (91) but does not interfere with other promoters. Tissue-specific promoters appear to retain specificity (92,93) and a number of other regulated expression systems have now been used successfully in AAV vectors (94–96). Introns function in AAV vectors and may enhance expression, and more than 1 promoter and gene cassette can be inserted in the same vector. Importantly, transcription from AAV does not seem to be susceptible to in vivo silencing, as shown by expression for over 1 year after intramuscular delivery in rodents (54,55,97).

B. Production of AAV Vectors

AAV has one of the largest burst sizes of any virus, as noted above, and this implies that a high yield of AAV vector particles per cell theoretically is attainable (7). Maximizing the specific productivity may avoid unnecessary increases in biomass during vector production. However, the cytostatic properties of the AAV rep protein presented an obstacle to generation of stable packaging cell lines for producing AAV vectors (72). Consequently, AAV vector production initially was based on transient transfection of a vector plasmid and a second plasmid, to provide complementing rep and cap functions, into adenovirus-infected cells, usually the transformed human 293 cell line. The original vector production systems yielded a mixture of AAV vector and adenovirus particles, and exhibited relatively low specific productivity (6,7). Furthermore, recombination between the vector plasmid and complementing plasmids generated wild-type AAV (AAV), pseudo wild-type or replication-competent AAV (rcAAV), or other recombinant AAV species (98,99).

Upstream production of AAV vectors can now be accomplished by DNA transfection-based methods in which adenovirus infection is replaced by DNA transfection with the relevant adenovirus genes or by cell-based AAV production systems that do not require DNA transfection. Both the transfection and cell-based approaches can give a specific productivity in excess of 10^4 vector particles per cell. However, the cell-based systems are probably more amenable to scale-up for commercial production than are DNA transfection systems. Downstream processing and purification are now usually accomplished by chromatographic procedures that give a higher degree of purity than the earlier purification procedures using ultracentrifugation.

C. Complementation Systems

Three approaches have been taken with respect to upstream production of AAV vectors. First, in DNA transfection-based procedures, various modifications have been made to the complementing *rep-cap* gene cassette in an attempt to enhance specific productivity and to decrease production of rcAAV. One group demonstrated that expression of rep and cap proteins may be limiting (100), but 2 other studies (101,102) suggested that cap proteins were limiting due to down-regulation of cap by increased production of rep. A packaging plasmid that has the Rep78/68 expression down-regulated by changing the initiation codon AUG to ACG was reported to give higher cap expression and higher yields of vector particles (102).

The only adenovirus genes required for full helper function are E1, E2A, E4, and VA, and transfection of the latter 3 genes into cells that contain the E1 genes, such as human 293 cells, can provide full permissivity for AAV (68). The infectious adenovirus can be replaced as the helper with a plasmid containing only the adenovirus E2A, E4, and VA genes (103,104) that, together with the E1A genes supplied by 293 cells, provide a complete helper function in the absence of adenovirus production. Another group (105) used a plasmid containing nearly all the adenovirus genome except the E1 region, but this yielded infectious adenovirus, probably by recombination with the E1 region in the cell. All these systems require transfection with 3 plasmids, for vector, rep-cap, and adenovirus helper function, respectively. In contrast, Grimm et al. (106) combined all 3 adenovirus genes and the *rep-cap*

genes into a single plasmid. In general, all these approaches increased vector productivity compared with earlier systems such as pAAV/Ad (107), and productivities of at least 10^4 particles per cell have been reported. Nevertheless, these approaches still require DNA transfection and may be unwieldy for production scale-up.

An alternate approach to AAV vector production is to generate stable cell lines that contain the *rep* and *cap* complementing genes, the vector genome, or both. To avoid DNA transfection, the cells must still be infected by a helper virus, adenovirus, but this can be removed readily as a result of advances in downstream purification processes (see Section VI.E). Rescue of vector from a producer cell line having the vector stably integrated was demonstrated by transfecting the cells with a rep-cap helper plasmid and infecting with adenovirus (108). Stable cell lines containing a *rep* gene capable of generating functional rep protein were constructed by Yang et al. (72) who replaced the p_5 promoter with a heterologous promoter. Clark et al. (109) generated cell lines containing the *rep* and *cap* gene cassettes but deleted for AAV ITRs. Furthermore, the vector plasmid could be stably incorporated into the packaging cells to yield AAV vector producer cell lines (109,110). Producer cell lines provide a scalable AAV vector production system that does not require manufacturing of DNA and may reduce generation of rcAAV. However, a new producer cell line must be generated for each individual AAV vector and this may be laborious.

A modification of the packaging cell line method is to use a cell line containing a *rep-cap* gene cassette that is then infected with an Ad/AAV hybrid virus. The Ad/AAV hybrid is an E1 gene-deleted adenovirus containing the AAV ITR vector cassette (111,112). After infection of cells containing the *rep-cap* genes, the AAV-ITR cassette is excised from the Ad/AAV, amplified, and then packaged into AAV particles. This allows the same packaging cell line to be used for production of different AAV vectors simply by changing the Ad/AAV hybrid virus, but it requires coinfection with adenovirus to provide the E1 gene function. It is worth noting that the Ad/AAV hybrid viruses can also be used as delivery vehicles for AAV vectors (113–115), but this might suffer some disadvantages such as induction of innate immune responses characteristic of the adenovirus capsid interaction with cells. Another packaging cell system was described (116) in which the packaging cell contains both a *rep-cap* gene cassette and the AAV ITR vector cassette, and both cassettes are attached to an SV40 replication origin. Also in the cells is a SV40 T antigen gene that is under control of the *tet*-regulated system such that addition of doxycycline induces T antigen, which in turn results in amplification of the *rep-cap* and the vector cassettes. Subsequent infection of the cells with adenovirus renders the cells permissive for vector production.

Herpes simplex virus also can be used in production of AAV vectors by generating 2 types of HSV/AAV hybrid viruses. One approach (117,118) uses an HSV/AAV hybrid virus in which the AAV *rep-cap* genes, under control of their native promoters, were inserted into the HSV genome. This HSV/AAV *rep-cap* virus could generate AAV vector when infected into cell lines along with a transfected AAV vector plasmid or into cell lines carrying an AAV vector provirus. Alternatively, an HSV/AAV hybrid virus was constructed by inserting an AAV ITR vector cassette between HSV genome replication origins and then packaging this construct into an HSV particle using an HSV amplicon system (119,120). These HSV/AAV vector hybrids were infectious for neural cells, but the biological properties and fate of these HSV/AAV hybrid genomes after infection of cells is complex and not yet well understood. Finally, insect cells can also be used to produce AAV vectors. In this system, baculovirus vectors containing *rep-cap* gene cassettes or the AAV-ITR vector cassette are used to infect insect cells and AAV vectors can be generated (121).

D. Replication-competent or Wild-type AAV

Wild-type AAV is not a human pathogen but generation of wild-type or rcAAV during vector production needs to be avoided for several reasons. The presence of wild-type AAV in vector preparations may increase the likelihood of vector mobilization following a helper virus infection in the patient, which could increase the likelihood of cellular immune responses to AAV proteins and can cause significant alterations in the biology of the vector because of the pleiotropic effects of the rep proteins. The earliest AAV vectors (4,5) were produced by cotransfection with helper plasmids that had overlapping homology with the vector, and this generated vector particles contaminated with wild-type AAV due to homologous recombination. Reduction of the overlapping AAV sequence homology between the vector and helper plasmids reduced, but did not eliminate, generation of wild-type AAV (98,107,108).

A combination of vector plasmid and packaging plasmid in which the AAV region containing the P_5 promoter was not present in either plasmid prevented generation of wt AAV but some pseudo wild-type AAV (rcAAV) was generated at very low frequency by nonhomologous recombination (98,108). This nonhomologous recombination was decreased to undetectable levels in a packaging system (split-gene packaging) carrying *rep* and *cap* genes in separate cassettes (98) so that 3 or 4 recombination events would be required to generate rcAAV. An alternate approach to decreasing pseudo wild-type or recombinant AAV is to insert a large intron within the rep gene in the helper plasmid so that any recombinants would tend to be too large to package in AAV particles (122).

It is likely that all vector production systems may have a propensity to generate pseudo wild-type AAV or other recombinants of AAV at some low frequency because it is not possible to eliminate nonhomologous recombination in DNA. This may be more likely in DNA transfection systems, especially in view of the very large genome numbers that are normally introduced into transfected cells. Thus, packaging systems in which transfection is avoided may help to reduce the frequency of such recombination. Standardized assays to analyze and evaluate such rcAAV or recombinant species in AAV vector preparations have not yet been developed. However,

to detect replication competent species, an assay that employs 2 cycles of amplification in non-*rep*/*cap* expressing cells, and then a sensitive readout such as hybridization or PCR (rather than rep or cap immunoassays), is likely to be required.

E. Purification

Historically, AAV was purified by proteolytic digestion of cell lysates in the presence of detergents followed by banding in CsCl gradients to concentrate and purify the particles and separate adenovirus particles. Significant progress has been made in the downstream processing of AAV vectors, and this has led to much higher quality and purity. This is critically important for preclinical studies and clinical trials. The reliance upon the original CsCl centrifugation techniques is being abandoned because it is a cumbersome procedure that does not provide high purity, it may inactivate some AAV vector, and it is difficult to envision its use for commercial production. Several groups have employed nonionic iodixonal gradients as an initial bulk-recovery method (123,124). A variety of chromatographic methods, including ion exchange resins and antibody, heparin, or sialic acid affinity resins, in both conventional and high-performance liquid chromatography formats, have been employed (106,125–134). Chromatographic procedures will be generally more acceptable for the biopharmaceutical manufacturing as therapeutic applications are developed for AAV vectors.

F. Assay of AAV Vectors

Characterization and testing of AAV vectors are critical elements for clinical development. Although there is variability in assay methods and properties measured, the minimal testing should include measure of vector strength by determining the concentration of capsids containing vector genomes, a measure of total capsids, a measure of ''infectivity'', and an assessment of vector purity. The efficiency of transgene expression or potency of the vector should also be measured. In some cases, it is difficult to measure potency using in vitro assays because transgene expression may be mediated by weak or tissue-specific promoters.

Early in the development of AAV vectors, the concentration was measured using dot-blot hybridization of vector genomes and comparing the signal intensity with a standard curve derived from a plasmid containing the vector genome (135). Recently, more precise and quantitative assays have largely supplanted the dot blot assay. Measurement of vector concentration by real-time PCR amplification (TaqMan) of the vector genome has resulted in increased precision and accuracy (126). These assays measure the number of vector genomes that are encapsidated and thus protected from digestion by DNAse. Consequently, vector concentration is expressed in units of DNAse-resistant particles (DRPs). This type of assay can be used to facilitate reliable quantitation of vectors during process development for production of multiple AAV serotypes (118).

Infectivity measurements of vector preparations are important mainly to establish a consistency of vector manufacturing rather than being an indication of vector potency in vivo. By their nature, AAV vectors are replication defective, so in vitro measurements of infectivity are artificial and generally require complementation with *rep* and *cap* as well as helper virus functions. Initially, vectors were measured by an infectious center assay in which wtAAV as a source of rep and cap and adenovirus were added, along with dilutions of vectors to infect cells (136). Cells that were replicating the vector genome were then scored by probe hybridization to individual cells collected on a filter, hence the term ''infectious center''. The development of cell lines containing rep and cap (137) has allowed infectivity measurements of vectors to be performed in a 96-well format in an assay that yields data with higher precision than the infectious center assay, and is amenable to a high-throughput format that can be used for purification development (138). In conjunction with the real-time PCR assay, this type of infectivity assay yields data on particle to infectivity (P:I) ratios of vectors with high precision, thus aiding process development. It is important to recognize that the apparent particle to infectivity ratio of vectors based on different serotypes of AAV may be quite different due to the natural variation that exists in receptor usage and potentially in intracellular trafficking among the serotypes. A cell line containing rep and cap may be more efficiently transduced by one serotype as compared with a second serotype, yielding differences in P:I ratios that may not be reflective of differences in vector potency in vivo but rather a difference in the ability of a given serotype to efficiently infect the *rep*/*cap*-expressing cell line used in the assay.

Other aspects of vector preparations are also worth measuring. These include a measure of the ratio of empty to full capsids such as a capsid enzyme-linked immunosorbent assay (139), negative-stain electron microscopy analysis (131,132), or a measure of total protein compared with concentration of genome-containing vectors and an assessment of vector purity, usually by acrylamide gel electrophoresis analysis. For clinical development it is important to measure the residual contaminants, including host cell protein, DNA, and serum components. In cases where the vector is manufactured from a cell line of tumor origin (e.g., HeLa cells), determination of the residual level of host cell DNA is an important part of the release testing. If the vector is of high purity, its aggregation state can be assessed by using dynamic laser light scattering. This method enables a measurement of the hydrodynamic radius and provides a size distribution of particles in solution and the relative amount of particles in each size class. This measure is useful for vectors at high concentration and can aid in development of formulations that maintain vectors in a monomeric, nonaggregated state.

VII. GENERAL PROPERTIES OF AAV VECTORS

A. Serotypes of AAV

Growing appreciation of natural variation among AAV serotypes, as well as its application to vector production and usage,

has added to the attractiveness of rAAV as a gene therapy modality. There are currently 8 serotypes of AAV described with the complete genome sequence information available for AAV1–AAV6 (22–27,30) and capsid gene sequence for AAV7 and AAV8 (31). AAV6 has been shown to be a recombinant between AAV1 and AAV2 and is not a distinct serotype because it is neutralized by anti-AAV1 antiserum (140). In addition, there are two isolates and sequences of AAV3 that differ from each other in a number of amino acids in both rep and cap (30). Table I summarizes current knowledge of receptor usage by these viruses (42–44,141,142). This variation in receptor usage is at least in part responsible for the increasingly appreciated efficiency of vectors of different serotypes to transduce specific cell types and tissues in vivo. The serotypes of AAV are highly related to each other with the notable exception of AAV5 (26,27). AAV5 is the most distantly related of the serotypes, and also displays a serotype-specific terminal resolution site (trs) in its ITR (143). Even though rep proteins from other serotypes will bind the AAV5 ITR, they do not efficiently cleave at the trs.

Most early studies with AAV vectors used serotype 2. A number of studies demonstrated transgene expression in muscle, liver, central nervous system (CNS) tissues, lung, and retina (51–55,75–78,92,93,97), with persistence for months to years. As other serotypes of AAV have been used in vector production, it has become apparent that there are distinct and often advantageous differences among vector serotypes in their efficiency of transgene expression in various tissues and cell types. It is difficult to list a hierarchy of transduction efficiencies of target organs relative to individual serotypes because there are conflicting data in the literature both with regard to organ tropism and the threshold of expression differences reported from different laboratories. These differences may reflect differences among strains of mice used in the studies or differences in vectors produced by different investigators. However, some general conclusions can be made. For example, AAV1 and AAV7 appear to be superior, by several orders of magnitude in transgene expression, to other serotypes for transduction of murine muscle tissue, but AAV5 vectors also demonstrate enhancement compared with AAV2 vectors (31,144–146). AAV8 is the most efficient serotype for murine liver transduction (31) being up to 100-fold higher than AAV2 vectors, and AAV5 appears superior in transduction of cells in the murine respiratory tract (147,148). AAV5 generally appears to be superior to AAV2-based vectors in all tissue types so far tested, including CNS, muscle, liver, and retina (144–146,148–150). Similarly, AAV6 vectors are more efficient than AAV2 in transducing murine airway epithelia and alveoli, whereas AAV3 vectors are superior in transducing smooth muscle cells (151,152).

The increased transduction efficiency noted for AAV5-based vectors in mouse airway may be due in part to the demonstrated increase in binding to the apical surface of polarized airway epithelial cells, suggesting that receptors are an important part of vector tropism (46,147). However, trafficking of vectors also plays a role in transduction efficiency (50). Approximately 5% of hepatocytes are transduced following delivery of AAV2-based vectors to mice (153), in spite of the fact that every cell contains vector DNA 1 day following administration. This points out that events postbinding and internalization are important for successful transduction (see below). Interestingly, when vectors based on AAV5 or AAV8 were administered to mice, increased numbers of hepatocytes were transduced (>15%) and increased levels of transgene expression were observed (31,150).

AAV4 vectors transduce ependymal cells in the murine CNS almost exclusively, whereas AAV5 vectors transduce both neurons and astrocytes (149). In retina, a number of studies have demonstrated large differences among serotypes in the ability to transduce photoreceptor cells and the retinal pigmented epithelium (141,154,155).

Overall, there is a great variability among different AAV serotypes in their ability to transduce various cells, tissues, or organs efficiently, depending on the route of administration and the capsid type. Further work will be needed to understand the relative importance of the factors contributing to differences in transduction efficiencies with different serotypes of AAV vectors.

It may be possible to exploit this natural tropism variation to target transgene expression to specific organs and/or cell types by selecting the appropriate capsid for vector production. The increase in transduction efficiency among serotypes should also allow transgene expression to be achieved with a decreased vector dose. This may have importance for decreased cost of goods in manufacturing therapeutics and decreased antigenicity of the vector dose.

Table 1 Cellular Receptors for AAV Serotypes

Serotype	Primary receptor	Secondary receptor
AAV1	Unknown	Unknown
AAV2	Heparin sulfate	$\alpha_\gamma\beta_5$ Integrin/FGFr
AAV3	Heparin sulfate	Unknown
AAV4	O-linked sialic acid	Unknown
AAV5	N-linked sialic acid	PDGF receptor
AAV7	Unknown	Unknown
AAV8	Unknown	Unknown

VIII. VECTOR METABOLISM

Early studies of AAV vectors led to the suggestion that the major factor limiting the efficiency of transduction was the process of converting the ss genome to a duplex molecule. More recent studies are beginning to reveal a complex pathway of events that impact the function of AAV vectors. Thus, delivery of the AAV vector genome to the cell nucleus may be influenced by the availability of specific receptors and coreceptors for AAV binding and entry, and by cellular trafficking, including a potential diversion of AAV particles into a ubiquitin-mediated proteosome degradation pathway. After the ge-

nome is successfully delivered into the nucleus, a succession of events results in the vector genome becoming transformed into larger concatemeric molecules. Most of these concatemeric molecules appear to be maintained for prolonged periods, perhaps for the lifetime of the cell, in the nucleus as episomal molecules; very few, if any, integrate into the host genome.

A. Cellular Binding and Trafficking of AAV Vectors

The ability of a vector particle to interact with a specific receptor molecule on a target cell is critical for successful transduction. Table I lists currently identified primary and secondary receptor molecules for AAV serotypes. The primary receptors identified (heparin sulfate and sialic acid) are commonly found on many cells and are also the receptor molecules used by a large number of viruses besides AAV. This suggests that additional receptors that lend more specificity to attachment and penetration of cells might exist, and several such coreceptors have been identified (Table I).

Although attachment is a critical first event, it does not necessarily imply that the vector will be able to efficiently transduce the cell. This has been increasingly apparent in recent years as a more detailed understanding of the trafficking and uncoating of AAV vectors has been accumulated (46–50). For example, polarized human airway epithelial cells are transduced with varying efficiencies by AAV2-based vectors, depending on the route of delivery and entry from the basolateral surface, results in about a 200-fold increase in gene expression in the cells compared with vector administered from the apical surface (46,156). Surprisingly, the difference in vector attachment to the 2 cell surfaces is only about 5-fold. This finding led to the discovery that the vectors traffic differently in these cells, depending on the side of the polarized airway to which they bind (46,156). Vectors administered from the apical surface are modified by ubiquitination. The addition of proteosome or ubiquitin-ligase inhibitors led to an increase in transduction following apical administration of vectors. These comparisons of differences in binding and differences in transduction efficiency have led to an appreciation that cellular trafficking of vectors is important to successful transduction, and that it may differ between cell types and even within individual polarized cells between the apical and basal surfaces.

After binding to the cellular receptor, AAV2 is internalized by an endocytosis mechanism that for heparin binding appears to be mainly via clathrin-coated pits (157,158), although some clathrin-independent uptake may also occur (157,158). Additional studies using fluorescent Cy3-conjugated AAV2 vector particles showed that endocytosis can be mediated by an $\alpha_v\beta_5$ integrin/Rac1-dependent mechanism and that subsequent trafficking to the nucleus requires activation of PI3K pathways, as well as functional microtubules and microfilaments (160). Evidence for the involvement of early endosomes in AAV trafficking, as well as the involvement of microtubules and microfilaments, exists mainly from the use of inhibitors (48,49). There is a growing consensus that, in the absence of helper virus, there is a block at the step of import of viral vector genomes into the nucleus of the cell (161–163). However, there is no clear understanding as to how or where uncoating of the viral genome occurs, or how the vector enters the nucleus, although it does not appear to use the nuclear pore complex for this step (163,164).

B. Vector DNA Metabolism

For rAAV vectors to transduce a cell, the ss DNA genome must be converted into a double-stranded form (33), as shown in Fig. 3. Several reports, based on transduction of cells in vitro, indicated that this single- strand to double-strand (DS) conversion step might be rate limiting in the absence of helper virus coinfection (165–167). The adenovirus E4 ORF6 protein has been shown to increase the level of transgene expression and to increase second-strand DNA synthesis (168). More recently, a cellular protein that bound to the single-stranded D-sequence of the AAV ITR was identified as the well-known FK506-binding protein (FKBP-52). This protein has been implicated in controlling the conversion of ss to ds DNA in vector-infected cells and phosphorylation of FKBP-52 influences its ability to bind the D sequence (169,170). When phosphorylated at tyrosine residues (by the epidermal growth factor receptor protein tyrosine kinase), FKBP-52 binds to the ss D sequence region of the ITR that is present in infecting vector genomes, and second-strand DNA synthesis is impaired. The efficiency of transduction in a number of cell types in vitro and in vivo correlates with the phosphorylation state of FKBP-52. In HeLa cells, overexpression of a cellular phosphatase (TC-PTP) that can use the FKBP-52 protein as a substrate, led to dephosphorylation of the FKBP-52, an increase in AAV second-strand DNA synthesis, and an increase in transgene expression. Transgenic mice expressing either the wt or a catalytically mutant form of the phosphatase were created. Hematopoietic stem cells from transgenic mice expressing the wt TC-PTP phosphatase were transduced by an AAV2 vector, but those from mice expressing the phosphatase-negative mutant were not. These results suggest that the block to second-strand DNA synthesis is due to binding of FKBP-52 to the D-sequence of infecting vector genomic DNA and that this binding is regulated by phosphorylation.

A second mechanism for conversion of ss DNA to ds DNA has been proposed. In this model, annealing of negative and positive sense ss DNA genomes (both of which are efficiently packaged in AAV particles) occurs in cells to form ds DNA in the absence of second-strand DNA synthesis. There is some experimental evidence for this self-annealing model both in vitro and in vivo (171), although it is not consistent with observations that AAV infection displays single-hit kinetics (59). However, it is possible that double-stranded DNA may be formed via either pathway in cells transduced with rAAV vectors.

Further support for the idea that conversion of single-stranded vector genomes into transcriptionally active, double-stranded forms is crucial for transduction and is a rate-limiting step comes from a study demonstrating that vector genomes

smaller than half the size of the AAV genome are packaged in multiple ways (172). Particles contain either a single vector genome, two copies of the small vector genome, or a covalently linked double-stranded hairpin molecule equivalent to a replicative-intermediate formed during vector genome replication. In a separate study, vectors containing these small self-complementary genomes were shown to be relatively insensitive to the enhancing effects of adenovirus on transduction, to be resistant to the effects of DNA synthesis inhibitors, and displayed altered kinetics of transgene expression in vivo (173). When these vectors were administered to mice, an increase in transduction from the usual 5% to more than 50% of hepatocytes was observed (174,175). These results strengthen the model that second-strand DNA synthesis is a rate-limiting step for transduction, at least in hepatocytes.

Once the vector genome is converted into a double-stranded DNA molecule, a number of fates have been reported. One group of studies convincingly demonstrates that the viral genome circularizes, and that these circular monomers recombine at the AAV ITRs to form larger circular concatemers of head-to-head, head-to-tail, and tail-to-tail arrangements both in vitro and in vivo (79,80,176,177). These genomic concatemeric molecules, containing a ''double-D'' ITR structure (an ITR bracketed on each side by the D sequence and presumably formed by an ITR-ITR recombination event) persist extra chromosomally as episomes and are responsible for the long-term persistent transgene expression seen with rAAV vectors. Recently, it was reported that severe combined immunodeficiency (SCID) mice that are deficient in DNA-dependent protein kinase activity (DNA-PK) lack the ability to convert rAAV genomes into circular concatemers (178). Rather the concatemers formed in these mice appear to be linear molecules, suggesting that DNA-PK activity is involved in the formation of circular episomes. In both cases (normal and SCID mice) transgene expression persisted for 1 year at similar levels. A second group of reports (171,179,180) suggest that vector genome concatamerization occurs by recombination of linear monomeric genomes. It is possible that both mechanisms of concatamerization are operative and one pathway is more likely to occur than another in a tissue-specific manner (e.g., muscle vs. liver). Despite the differences reported on the substrate for concatemer formation, it is clear that long-term transgene expression is mainly mediated by episomal concatemers of viral genomes rather than integrated molecules (83,84).

Evidence that DNA repair and recombination are directly involved in circularization or concatamerization of AAV vector genomes is supported by recent insights into possible biochemical mechanisms of their formation (178,180). In fibroblasts from a patient with ataxia telangiectasia (ATM), there is greatly enhanced formation of AAV vector circular forms and enhanced integration of the head-to-tail concatemers as proviral genomes (181). The ATM gene is a PI-3 kinase that regulates the p53-dependent cell-cycle checkpoint and apoptotic pathways, and in these ATM cells the DNA double-strand break (DSB) repair systems that normally can be activated by UV irradiation appear to be already activated maximally. Consequently, AAV vectors in these cells yield a high level of transduction and this is not activated further by UV irradiation (181), in contrast to the observations in normal cells (165–167). Additional evidence that DSB repair pathways are involved in regulating AAV transduction comes from observations that the proteins Ku86 and Rad52, which are known to recognize DNA hairpin structures and DNA termini and to promote repair of DSB, could associate with the AAV DNA ITR (182).

C. Dual Vectors

The ability of AAV vectors to form concatemers and the evidence that intermolecular recombination is directly involved in their generation has been used to extend the capacity of AAV vectors beyond the 4.5-kb payload limit in the dual-vector system. It is now possible to divide a gene expression cassette that is up to 9 kb in size between 2 AAV vectors and, following coinfection with both vectors, take advantage of the intermolecular recombination to generate the intact expression cassette. This process has been demonstrated both in vitro and in vivo, and shows remarkable efficiency (179,183–185).

Both *cis*-activation (183) and *trans*-splicing (184) modes of dual vectors have been described. In *cis*-activation, one vector carries a high-efficiency enhancer of transcription, and the other vector carries the transcription promoter and the gene sequence to be expressed. Recombination after infection places the enhancer in *cis* with the expression cassette and increases transcription. In the *trans*-splicing mode, one vector carries the transcription promoter and the 5′ part of a gene, and the other vector carries the 3′ part of the gene. By judicious arrangement of splice donor and acceptor sites, the appropriate mRNA can be derived from the read-through transcript using the heteroconcatemeric template. Interestingly, the intervening ITR sequence does not appear to inhibit read-through transcription or RNA splicing (183,184). It does appear, however, that in these dual-vector constructs, some transcription may be seen directly from the ITR, either because of its own promoter activity or because of enhancement from the *cis*-acting elements provided by the second vector (183,184).

Cis-activation was demonstrated using a vector containing super-enhancer elements comprising parts of the SV40 and CMV enhancers, together with a second vector containing the luciferase gene. This dual enhancer combination gave robust expression of luciferase in mouse skeletal muscle at levels that compared well with that from administration of a single vector expressing the same elements (183). In another study, 2 vectors containing a LacZ reporter cDNA or an enhancer promoter cassette from human elongation factor EIFα, respectively, were shown to yield reporter gene expression in livers of mice after dual administration by portal vein injection (179).

Trans-splicing was demonstrated using one vector that contained a transcription promoter and the 5′ part of the erythropoietin (epo) genome locus, and a second vector that contained the 3′ region of the epo locus (184). Dual injection of the 2 vectors into mouse skeletal muscle provided therapeutic

levels of epo sufficient to protect the mice from adenine-induced anemia. Similarly, another group used 2 vectors, one containing the CMV promoter with the 5′ half of the LacZ gene and the second containing the 3′ half of the LacZ gene (185). These vectors also gave robust expression of intact LacZ after dual injection into mouse skeletal muscle.

Recently, the dual-vector approach to packaging and expressing large genes in rAAV vectors was extended to an investigation of the use of homologous recombination between 2 vectors, each containing an overlapping DNA sequence to drive recombination (186,187). The data demonstrated that non-ITR-mediated recombination between 2 genomes can occur and result in expression of a large transgene. However, the efficiency of this homologous recombination process may vary, depending on the tissue type. In mice, the efficiency in muscle was low and yielded less than 1% of the level of expression seen from an intact vector, whereas in lung the efficiency approached that seen with an intact vector construct.

The dual-vector approaches to packaging and expressing larger transgenes in AAV particles offer a way to almost double the payload capacity of AAV vectors that should extend the utility of this vector system to larger genes. Careful analysis will be required to determine additional safety issues or risks this approach might present for clinical use. For instance, for some applications such as hemophilia A, it may be deleterious to express a partial FVIII protein. Nonetheless, as these systems are better characterized they may well prove to have clinical utility.

D. Capsid Modifications and Targeting of Vectors

There is an interest in modifying the capsids structure of AAV vectors to target transgene expression to a specific cell type (18). In general two approaches have been taken. In one approach, retargeting was obtained by the use of a bispecific antibody having one arm specific for AAV capsids and the second arm specific for a receptor on a cell (188). Although not involving capsid modifications, this method did achieve vector targeting to the specificity of the antibody used. The other approach to targeting involves alterations or additions to the amino acid sequence of the capsid proteins directly. With capsid protein modifications, the goal is to eliminate the natural receptor interactions of the capsid and replace it with a receptor interaction that would be engineered into the capsids as an amino acid sequence or receptor ligand. There are a number of studies that describe such attempts at vector retargeting, and some recent reports describe a systematic analysis of insertions into the capsid and the effect on vector yield and tropism (189–192). It is possible to generate vector particles with modified tropism as a result of amino acid substitution or addition to the capsid, and further work will reveal the usefulness of this approach for clinical gene therapy applications. Peptide ligands specific for various receptors have been successfully engineered into AAV2 capsids (189,191, 193–195), with the resultant vector target specificity being achieved. The recent determination of the crystal structure of AAV2 should aid these attempts in the future because positions of loops on the surface of the particle have been mapped along with residues responsible for interaction with heparin sulfate (17).

IX. HOST RESPONSES AND TOXICITY

Host immune responses, including innate immune responses, cellular immunity, or humoral antibody responses, may hinder the use of some gene therapy vectors. However, administration of AAV vectors has not been reported to induce innate immune responses or proinflammatory cytokines. Also, AAV vectors are replication defective and contain no viral genes, so cellular immune responses against the viral components should not be evoked readily. In all the in vivo studies of AAV vectors in rodents, rabbits, or rhesus macaques, there is little evidence of cellular immune responses to viral components.

AAV vectors may be used mainly for clinical applications requiring only infrequent delivery, but potential humoral immune responses against the viral capsid, either preexisting in the human population or induced by vector administration, must be considered (3,18). Reinfection of humans by AAV is not prevented by serum neutralizing antibodies (3,18). However, more extensive studies will be required to assess whether induction of neutralizing antibody responses will pose any limitations to AAV vectors.

Induction of anti-AAV capsid antibody responses after vector administration may reduce the efficiency of transduction upon readministration (54,196,197). This depends on the route of administration (73,198,199) and also may depend on the quality of the vector preparations. In one study in which 2 AAV vectors expressing the reporter genes bacterial β-galactosidase or human alkaline phosphatase were successively administered to lungs of rabbits, expression from the second administered vector was impaired and this was ascribed to a neutralizing antibody response (200). Similar studies in mice also implied that neutralizing antibodies impaired readministration of AAV vectors, but that this could be partially or completely overcome by transient immunosuppression with anti-CD40 ligand antibodies or soluble CTLA4-immunoglobulin at the time of the initial vector administration (197,201). However, interpretation of such studies may be complicated by the expression of the foreign reporter proteins that could represent confounding variables. Furthermore, other studies showed that immune responses were greatly reduced following airway administration of AAV (73) and that vector transduction could be seen after at least 3 repeated administrations to the lung of rabbits (32). Thus, up to 3 successive administrations of AAV vectors to rabbit lungs over a 20-week period did not prevent gene expression from the third delivery of vector (32).

It remains to be determined how neutralizing antibody responses to AAV vector capsids might impact applications of AAV vectors. This will most likely require studies in humans

to determine if the various animal models such as rodents or rabbits are predictive for the immune response to AAV vectors in humans and whether such immune responses will pose any limitations to their therapeutic application. For relatively infrequent administration of AAV vectors, transient immune blockade (196,197) may not be an attractive option for therapeutic use of AAV or any other gene delivery vectors. An alternate possibility might be to use vectors that have capsids of different serotypes for subsequent administrations (152).

Immune responses to the transgene expressed by an AAV vector vary and may depend on the route of delivery. Both MHC class II-restricted antibody responses and MHC class I cytotoxic T lymphocytes have been reported, but this may vary with the route of administration (199). In some studies, such as intramuscular delivery in mice, there was no immune response to an expressed foreign reporter gene such as bacterial β-galactosidase, and it was suggested that AAV may be a poor adjuvant or may not readily infect professional antigen-presenting cells in muscle (202,203). However, an AAV vector expressing the herpes simplex virus type 2 gB protein was delivered intramuscularly into mice and elicited both MHC class I-restricted CTL responses against the gB protein and anti-gB antibodies (204). Following intramuscular delivery of an AAV human factor IX vector (205,206), there was an antibody response but not a CTL response against the FIX protein. The rules governing immune responses to foreign transgenes following AAV vector delivery remain to be elucidated more fully, but AAV may have utility as a viral vaccination vector (see Section X.H).

There have been few, if any, documented reports or indications of toxicity mediated by AAV vectors. The toxicity of AAV vectors has been extensively tested for a vector expressing the cystic fibrosis transmembrane regulator (CFTR) protein following delivery of these AAV-CFTR vector particles directly to the lung in rabbits and nonhuman primates. In rabbits, the vector persisted and expressed for at least 6 months, but no short- or long-term toxicity was observed and there was no indication of T cell infiltration or inflammatory responses (51). Similarly, in rhesus macaques, AAV-CFTR vector particles were delivered directly to one lobe of a lung and also persisted and expressed for at least 6 months (207). Furthermore, no toxicities were observed by pulmonary function testing, radiological examination, analysis of blood gases and cell counts, and differential in bronchoalveolar lavage or by gross morphological examination or histopathological examination or organ tissues (207). Studies in rhesus macaques were also performed to determine if the AAV-CFTR vector could be shed or mobilized from a treated individual (52). AAV-CFTR particles were delivered to the lower right lobe of the lung, and a high dose of adenovirus and wild-type AAV particles were administered to the nose of the animals. These studies indicated that the vector was not readily mobilized and suggested that the probability of vector shedding and transmission to others is likely to be low. The favorable safety profile of AAV in these preclinical studies has been predictive of a similar safety profile observed in clinical trials of the AAV-CFTR vector in CF patients (9–11). Importantly, preclinical studies of biodistribution of the AAV–CFTR vector following pulmonary delivery in rabbits and macaques showed that there was minimal spread of vector to organs outside the lung, no vector in gonads, and no toxicity was noted in any organ. However, use of different delivery routes may lead to more extensive biodistribution.

An AAV-FIX vector has been studied in preclinical rodent and canine models in support of clinical trials in hemophilia B patients via intramuscular injection or intravenous delivery via the hepatic portal vein. The vector showed a good safety profile when administered by intramuscular injection to hemophilia dogs that was reflected in the intramuscular injection clinical trial (12). However, in a second clinical trial, following intravenous hepatic administration of the AAV-FIX vector, patient semen was positive for the vector genome for a few weeks. This indicated that vector had apparently been distributed to gonads, although further investigation indicated that the vector was not present in sperm and the clinical trial has continued (208). A more extensive study of AAV-FIX vector delivery by both intramuscular and intravenous routes showed that, using a sensitive DNA-PCR assay, there was a dose-dependent detection of vector genomes sequences in the gonads of males of several animal species including mice, rats, rabbits and dogs (208). However, although testis tissue of these species was positive for vector for a short period after delivery, in both rabbits and dogs, semen and sperm were negative for vector sequences, suggesting that the risk of inadvertent germline transmission of vector sequences after intramuscular or intravenous delivery is extremely low (209). Two additional studies of AAV vectors expressing FIX (210) or α-1-antitrypsin (211) genes, after intramuscular delivery into rhesus macaques, again showed excellent safety profiles and did not detect transmission of vector sequences to gonads.

AAV has never been associated with any disease or shown to promote tumorigenesis. Indeed, several studies show that AAV or AAV ITRs can inhibit tumorigenesis (212,213). Also, recent studies in rhesus macaques infected with wild-type AAV2 by intramuscular, intravenous, and intranasal routes showed that, although there were some antibody responses against the AAV capsid, there was no cellular immune response to AAV components and no indication of any tumorigenesis (73). However, there was a recent anecdotal observation of tumors occurring in mice having a homozygous mutation in the β-glucuronidase gene, that were treated as neonates via head vein injection with an AAV vector expressing β-glucuronidase (BGUS). These mice exhibit the lysosomal storage disease mucopolysaccharidosis VII (Sly syndrome) that is characteristic of the human disease and, if left untreated, the animals die in several months. The mice treated with the AAV-BGUS vector survived up to 18 months and showed a remarkable biochemical and physiological correction of the disease (214). However, at 12 to 18 months some of these mice developed hepatic tumors, but vector genomes could not be found in all the tumors and not at any higher frequency than in nontumor tissue from the same animals (214). Thus, these tumors were probably not caused by insertional mutagenesis and clonal expansion. Furthermore, no

other studies of AAV vectors, including intravenous delivery in the same animal model and a variety of other animal disease models, have ever shown any tumor formation (214,216). The tumors in these MPSVII mice remains as an anecdotal observation because there were no sham-treated control mice that survived as long. It is possible that the tumor formation is specific for this disease, or the particular animal model, for overproduction of BGUS or to some other unknown cause and does not have any direct relation to the use of an AAV vector. However, some caution needs to be exercised and MPSVII disease possibly may not be an attractive candidate for gene therapy with an AAV vector.

X. AAV VECTOR APPLICATIONS

AAV vectors are now being developed for therapeutic applications. On the basis of extensive preclinical studies performed in rabbits and nonhuman primates, an AAV vector expressing the CFTR cDNA has been introduced into clinical trials in cystic fibrosis patients. This was the first AAV vector to undergo clinical trials that have now advanced to phase II (8–11). An AAV vector expressing factor IX is now undergoing phase I clinical trials in patients with hemophilia B (12).

In this section, we describe a series of therapeutic applications that are being developed with AAV vectors that either are now in clinical trials or are expected to enter clinical testing in the next several years. All the examples that we discuss are chronic diseases that affect various organ systems and require different routes of delivery. These examples serve to illustrate the potential wide applicability of AAV vectors, particularly for persistent gene expression in nondividing or slowly dividing cells.

A. Cystic Fibrosis

Cystic fibrosis (CF) is a lethal autosomal recessive disease that is caused by a mutation in the CFTR gene. The defect in the CFTR protein, which is a chloride ion channel expressed in epithelial cells, leads to complex biochemical changes in several organs, including lung and often the exocrine pancreas. In the lung, there is a decreased mucociliary clearance, increased bacterial colonization, and a chronic neutrophil-dominated inflammatory response that leads to progressive destruction of tissue in the conducting airways. The usual cause of morbidity and eventual mortality in CF patients is progressive loss of lung function. Thus, the goal of a gene therapy for CF is to deliver the CFTR cDNA into the epithelial cells in the conducting airways of the lung. An AAV vector expressing the CFTR cDNA has been introduced into clinical trials in CF patients by delivery of the vector to the lung and the nasal epithelium (8,10) in addition the maxillary sinus (9,11). One challenge in constructing an AAV vector expressing the CFTR protein was that the coding region requires a minimum cDNA size of 4.4 kb, thus leaving little space for a transcription promoter. To package this cDNA into a single AAV vector, advantage was taken of the discovery of the transcription promoter properties of the AAV ITR (91).

Although the lung is the target for a therapeutic gene therapy for CF, delivery to the maxillary sinus was undertaken as a novel attempt to obtain an early indication of the potential of the AAV CFTR vector. The maxillary sinuses of CF patients exhibit chronic inflammation and bacterial colonization that is reflective of some aspects of the CF disease in the lung. In addition, in CF patients who have undergone surgical bilateral anstrostomy, the maxillary sinus is accessible to instillation of vectors and for sampling and biopsy. In an initial trial, 15 sinuses of CF patients were treated with increasing doses of the AAV-CFTR vector (9). DNA PCR analysis of sinus biopsies showed dose-dependent delivery of the vector genome that persisted in the sinus for at least 70 days after instillation. In epithelial cell surfaces of CF patients, the transmembrane potential is hyperpolarized compared with normal patients because of the absence of a functional CFTR chloride channel. In the treated sinuses at the higher doses of vector, there was some reversal of the electrophysiological defect, thus providing suggestive evidence for expression of the CFTR protein from the delivered vector.

A follow-up, double-blinded, randomized, and placebo-controlled phase II study was performed in 23 CF patients in whom vector was administered to one maxillary sinus, while the contralateral sinus received a placebo treatment (10). This study confirmed the safety of the AAV-CFTR vector delivered to the sinus. In addition, the anti-inflammatory cytokine interleukin-10 showed a significant difference between vector- and placebo-treated sinuses over a 90-day period. This suggested that gene transfer could modulate levels of cytokines that may provide a useful surrogate marker for additional trials. Because several patients had participated in the previous phase I trial, the phase II study further suggested that the vector remains safe after multiple administrations to the sinus without induction of serum-neutralizing antibodies (10).

A phase I, single administration, dose escalation trial designed to assess safety and delivery of AAV-CFTR by inhaled aerosol to the lung was carried out in 12 adult CF patients exhibiting mild lung disease. The vector was well tolerated and no apparent safety concerns were demonstrated in the study. Vector administration at the highest dose of 10^{13} genome-containing particles resulted in significant gene transfer to the airway. A clear dose–response relationship was observed in vector gene transfer over 30 days, although the vector DNA declined over 90 days (11).

Following this study, a multidose, double-blinded, placebo-controlled, and randomized phase II trial was conducted in 37 CF patients with mild CF disease. Patients received 3 doses (10^{13} DRP per dose) at monthly intervals, administered by inhaled aerosol. This trial also was notable for enrolling patients as young as age 12 years, which is important because the eventual target population to treat CF is likely to be younger patients. The preliminary results of this study were presented recently (R. Moss, North American CF Meeting, Oct. 3, 2002, New Orleans). The multidose vector administration was safe and well tolerated. Interestingly, there was a significant treatment-related effect upon pulmonary function measured at 30 days and on the proinflammatory cytokines

IL-8 at 14 days. This is the first trial in CF patients to show an indication that gene delivery can positively affect a pulmonary function. Previous clinical trials in CF gene therapy had generally focused on measurement of molecular endpoints. However, clinical endpoints, such as pulmonary function that can be used for eventual approval of a successful therapeutic, are much more important.

Measurement of molecular endpoints, such as DNA PCR for vector genome delivery or gene transfer and RNA PCR or protein assay for gene expression, may be useful but in many cases it is difficult. Whereas these measurements can be performed readily in animal models on tissues obtained after necropsy, this generally is not an option in clinical trials except perhaps from autopsy samples, and even that is now uncommon.

For CF clinical trials, molecular endpoint measures have proven particularly challenging. First there is no good assay for CFTR protein. DNA and RNA PCR can be conducted on airways cells that are obtained via a brush inserted through a bronchoscope. This has several disadvantages including poor quality of the samples, which often contain very few cells. Consequently, it has been possible to obtain data from DNA PCR, but it has proven extremely difficult to obtain reliable RNA samples. In the clinical trials of the AAV CFTR vector in lungs of CF patients, gene transfer has been demonstrated but data on RNA expression has not been obtained. Other problematic issues are that the invasive bronchoscopy procedure disrupts other measures of pulmonary function and inflammation. Furthermore, the bronchoscopic procedure can only provide samples from the upper airways, whereas the vector needs to function in the lower airways, which is the primary site of the disease. In view of these considerations, measurement of inflammatory cytokines in sputum may be a less invasive way to obtain surrogate marker information and has the added advantage of reflecting events in the lower airways.

B. Hemophilia

Hemophilia is a severe X-linked recessive disease that results from mutations in the gene for either blood coagulation factor VIII (FVIII) in hemophilia A or IX (FIX) in hemophilia B. The absence of functional FVIII or FIX leads to severe bleeding diathesis. Expression of these clotting factors at less than 1% of normal levels results in severe disease, whereas levels over 5% of the normal range appear to be sufficient for normal function. Levels between 1% and 5% lead to much milder disease, and prophylactic delivery of these clotting factors at these levels of 1% of the normal levels (2–10 ng/mL and 50–250 ng/mL for FVIII and FIX, respectively) can decrease the risk of spontaneous bleeding into joints and soft tissues, and lower the risk of fatal intracranial bleeding. However, the FVIII and FIX proteins have a very short half-life, and this has stimulated interest in developing gene therapy approaches in which the clotting factors may be produced more persistently. Several groups have provided evidence that FIX protein can be expressed for prolonged periods in both murine and canine models after delivery of AAV-FIX vectors, either by portal vein injection to target the liver or by intramuscular injection (76,77,217–219). Generally, the levels of expression, as measured by the whole blood clotting time (WBCT) assay, have indicated that at least a partial correction of the defect in both the murine and the canine disease models can be achieved and suggest that accumulation of therapeutic amounts of FIX may be achievable in humans.

AAV vector delivery of the human factor IX gene into either immunodeficient or immunocompetent mice by portal vein injection into liver resulted in prolonged expression of factor IX for up to 36 weeks at serum levels of 250 to 2000 ng/mL, which is equivalent to about one-fifth of the normal human level (76). Similar portal vein delivery of human factor IX in hemophiliac dogs resulted in expression of factor IX at about 1% of normal canine levels, an absence of inhibitors, and sustained partial correction of whole blood clotting time (WBCT) for at least 8 months (217). In another study by Herzog et al. (76), intramuscular injection of an AAV human FIX vector into immunodeficient mice led to prolonged expression at about 350 ng/mL in serum for at least 6 months, but in immunocompetent mice there was generation of inhibitory antibody. These same investigators (218) subsequently showed that in hemophiliac dogs, intramuscular injection of high doses of the AAV-canine FIX vector achieved expression for over 17 months and demonstrated a stable, dose-dependent partial correction of the WBCT. Moreover, at the highest dose there was a partial correction of the activated partial thromboplastin time (APT). The APT assay may be a more reliable measure than WBCT. However, in another study (219), intramuscular injection of an AAV human FIX vector also led to a transient reduction of WBCT in the first week, but this was rapidly lost as the animals developed antifactor IX antibody.

Development of AAV vectors to deliver FVIII faces an extra challenge in that the FVIII protein cDNA is over 7 kb, which is too large to package into a single AAV vector. However, the FVIII protein is processed by excision of an internal B-domain such that the secreted protein consists of heavy and light chains. Removal of the B-domain DNA sequence reduces the size of the cDNA to about 4.2 kb, which can be packaged into a single AAV vector with space to include only small regulatory elements. This permits the generation of an AAV-FVIII vector expressing the human B-domain deleted FVIII that can correct the WBCT when injected via the tail vein into FVIII-deficient mice (220). An alternate strategy is to express the heavy and light chains from separate AAV vectors (221), but this would be less desirable for clinical application if it leads to unbalanced production of the two chains. Alternatively, use of dual *trans*-splicing AAV vector may also be feasible for expression of FVIII (222).

A clinical study of intramuscular injection of an AAV vector expressing human FIX in adults with severe hemophilia B was recently completed. Preliminary data from this study suggested that there was low-level expression of FIX in transduced muscle with no apparent formation of inhibitory antibodies. In addition, some possible modest changes in clinical endpoints were noted, including circulating levels of FIX and reduced frequency of FIX protein self-administration by 2

patients (12). A second phase I clinical trial with the same vector has been initiated in hemophilia B patients, but in this trial the vector is administered via portal vein injection to target the liver, which may be the natural source of FIX production. Clinical trials of AAV vectors in hemophilia A patients have not been initiated.

For hemophilia clinical trials, in contrast with the CF trials, a molecular endpoint is readily available because the level of the FIX or FVIII in serum can be directly measured and the level of protein will likely be related to degree of clinical benefit. In contrast, DNA or RNA measures again would require invasive biopsy, especially following portal vein delivery to the liver.

C. Muscular Dystrophy

The muscular dystrophies are a clinically and genetically heterogeneous group of disorders that show myofiber degeneration and regeneration, and are characterized by progressive muscle wasting and weakness of variable distribution and severity. They are associated with mutations in genes encoding several classes of proteins ranging from extracellular matrix and integral membrane proteins to cytoskeletal proteins, but also include a heterogeneous group of proteins including proteases, nuclear proteins, and signaling molecules [for a recent review, see (223)].

The most common myopathy in children, Duchenne muscular dystrophy (DMD), is a severe X-linked neuromuscular disease that affects approximately 1 of every 3500 males born and is caused by recessive mutations in the gene for the muscle protein dystrophin. Affected boys begin manifesting signs of disease early in life, cease walking at the beginning of the second decade, and often die due to cardiac arrest or respiratory insufficiency by age 20 years. The most common mutation in the dystrophin gene that causes the severe DMD phenotype is a deletion that results in a premature stop codon. The mutant protein is unable to bind to dystrophin-associated proteins at the muscle membrane. This lack of a functional dystrophin protein in DMD results in loss of muscle fiber integrity by disrupting the physical linkage between the actin cytoskeleton within the muscle fiber and the extracellular matrix (224).

Attempts to develop gene therapy for DMD have been complicated by the enormous size of the dystrophin gene, which is the largest known gene with a full-length cDNA that is 14 kb in size. However, dystrophin can retain significant function even when missing large portions of its sequence. For example, large, in-frame deletions in the central-rod domain often lead to the milder Becker muscular dystrophy (225). The *mdx* mouse is a naturally occurring murine model that has a premature stop codon generated by a point mutation in exon 23 of the dystrophin gene (226). Functional analysis of dystrophin structural domains in transgenic dystrophin-deficient *mdx* mice revealed multiple regions of the protein that can be deleted in various combinations to generate potentially highly functional minidystrophin genes (227). Three groups have generated functional miniature versions of the human dystrophin gene that can be readily packaged into AAV vectors (228–230). When injected into the muscle of *mdx* mice, efficient and stable expression was noted in a majority of myofibers, and the missing dystrophin and dystrophin-associated protein complexes were restored onto the plasma membrane. This treatment ameliorated dystrophic pathology in the *mdx* muscle and led to normal myofiber morphology, histology, and cell membrane integrity.

The limb girdle muscular dystrophies (LGMDs) are a heterogeneous group of inherited autosomal recessive neuromuscular diseases characterized by proximal muscular weakness and variable progression of symptoms. LGMD disease is caused by mutations in a number of genes, including 1 of the 4 small (cDNA $<$ 2 kb)-muscle sarcoglycan genes (α,β,γ,δ) expressed predominantly in striated muscle. These transmembrane glycoproteins associate with each other in equal stoichiometry to form the sarcoglycan complex, and a deficiency of one component typically leads to partial or complete absence of all the other sarcoglycan proteins on the sarcolemma. The Bio 14.6 cardiomyopathic hamster is a naturally occurring LGMD model due to a deletion in the δ-sarcoglycan gene. Administration of AAV δ-sarcoglycan vectors to these animals either by intramuscular or intravascular administration led to genetic, biochemical, histological, and functional rescue of relatively large regions of muscle (231–234). Mice that are null mutants for ψ-sarcoglycan exhibit severe muscle pathology that can be partly corrected if treated at less than 3 weeks of age by intramuscular injections of an AAV vector expressing the ψ-sarcoglycan gene from a muscle-specific promoter (235). Also, AAV α- or β-sarcoglycan vectors can rescue an α- or β-sarcoglycan defect in the corresponding knockout mouse model. Interestingly, while the β-sarcoglycan vector showed long-term sustained expression for more than 21 months and led to widespread biochemical and histological rescue of the dystrophic muscle, transduction of myofibers by the α-sarcoglycan vector was transient and correlated with induction of significant immune response (236). The transience of the latter vector was attributed to cytotoxicity resulting from overexpression, by more than 100-fold over normal levels of the protein rather than due to an immune response to the transgene. A phase I clinical trial of AAV to deliver sarcoglycan genes was initiated, but it enrolled and treated only 1 patient (12).

D. CNS Disease

Parkinson's disease (PD) is a common progressive neurodegenerative movement disorder that affects 5% of the population over 65 years of age. In this disease there is loss of dopaminergic neurons, mainly in the substantia nigra, which leads to deficiency of the neurotransmitter dopamine (DA) in the striatum. The clinical symptoms typically appear after extensive loss of 60% to 80% of the dopaminergic neurons has occurred, which is correlated with the DA deficiency. DA is synthesized from tyrosine, first through conversion to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH), and then to DA by aromatic amino acid decarboxylase (AADC). L-DOPA therapy is very efficacious, but response

declines as the disease progresses and is complicated by adverse side effects. Thus, the different enzymes involved in DA synthesis have been targeted for gene transfer replacement therapy in order to restore dopaminergic stimulation of the striatum.

Dopamine-deficient mice ($DA^{-/-}$), lacking TH in dopaminergic neurons, become hypoactive and aphagic and die by 4 weeks of age but can be rescued by daily treatment with L-DOPA. Bilateral coinjection of AAV vectors expressing human TH or GTP cyclohydrolase 1 (GTPCH1) into the striatum of these mice restored feeding behavior for several months. However, locomotor activity and coordination were only partially improved. A virus expressing only TH was less effective, and one expressing GTPCH1 alone was ineffective. TH immunoreactivity and DA were detected in the ventral striatum and adjacent posterior regions of rescued mice, suggesting that these regions mediate a critical DA-dependent aspect of feeding behavior (236).

Sustained and long-term transduction of striatal neurons and glial cells accompanied by significant behavioral recovery following administration of an AAV vector encoding the human TH cDNA has been demonstrated in the 6-hydroxydopamine (6-OHDA) rat model of PD (53). Also, using the 6-OHDA-lesioned rat model of PD, a single intrastriatal infusion of an AAV2-AADC vector led to an enhanced conversion of L-DOPA to restore DA to 50% of normal levels 12 weeks after vector administration (237). The vector-mediated increase in striatal decarboxylase activity remained undiminished over a 6-month period and persistence of transgene expression was demonstrated for at least 1 year (238). Coexpression of TH and AADC, using 2 separate AAV vectors in this Parkinsonian rat model, resulted in more effective DA production and more remarkable behavioral recovery compared with the expression of TH alone (239).

Convection-enhanced delivery of AAV2-AADC into 6 sites in the striatum of neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys restored AADC activity to levels that exceeded the normal range (240). Injection of a bicistronic AAV vector expressing genes for human TH and AADC to asymptomatic but dopamine-depleted monkeys that had been treated with MPTP demonstrated successful transduction of the primate neurons in the striatum over a period up to 2.5 months with suggestive evidence of biochemical phenotypic effects and without significant toxicity (241). Finally, triple transduction with AAV-TH, AAV-AADC, and AAV-GTPCH1 to the striatum of either 6-OHDA-lesioned rats or MPTP-treated monkeys has resulted in restoration of DA synthesis and motor function (242,243).

Another strategy for gene therapy of PD involves the use of neuronal-specific growth factors to regenerate or halt ongoing degeneration of dopaminergic neurons of the substantia nigra. Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic molecule for nigral dopaminergic neurons both in vitro (244) and in vivo (245). Using the 6-OHDA-lesioned rat model of PD, perinigral injection of an AAV vector encoding the rat GDNF 3 weeks prior to a striatal 6-OHDA treatment resulted in stable transgene expression for 10 weeks and significant protection of neurons from degeneration in the substantia nigra. However, there was no recovery of striatal TH-containing fibers and no recovery of motor function, suggesting that striatal dopaminergic recovery is necessary for functional improvement (246). In another study, both nigral and striatal long-term transduction of up to 6 months provided significant protection of nigral DA neurons against 6-OHDA-induced degeneration. However, only the rats receiving AAV-GDNF in the striatum displayed behavioral recovery, accompanied by significant reinnervation of the lesioned striatum. (247). In both studies (246,247), AAV-GDNF was administered before or shortly after the injection of the neurotoxin 6-OHDA. To better model the progressive DA depletion and significant neuron degeneration, which typically occur before symptoms appear, additional studies were carried out to examine the effect of AAV-mediated GDNF gene delivery into the striatum 4 weeks after injection of 6-OHDA. These studies showed that there was retrograde transport of GDNF to the substantia nigra that halted the ongoing degeneration of nigrostriatal DA neurons, with functional recovery, even after substantial numbers of DA cells had been lost (243,248,249).

Several clinical trials of AAV vectors in the CNS may begin soon. First, a novel approach to treatment of PD was proposed (250). In this clinical study, patients suffering from PD will be administered 2 different AAV vectors, each expressing, respectively, the gene for the 2 isoforms of the enzyme glutamic acid decarboxylase (GAD-65 and GAD-67) by coinjection into the subthalamic nucleus (STN) region of the brain. The STN, which has a central role in the region of the brain that is responsible for regulation of movement, becomes disinhibited in PD. Preclinical evidence suggests that the gene delivery can inhibit the STN.

A clinical trial to treat Canavan's disease, a childhood leukodystrophy, has been proposed (251). This disease results from an autosomal recessive mutation in the gene for aspartoacyclase, which causes a toxic accumulation of the metabolite N-acetyl-aspartate. In this trial, up to 21 patients ages 3 months to 6 years will be administered in up to 6 sites in the frontal, varietal, and occipital regions of the brain.

E. Ophthalmic Diseases

AAV vectors may be well suited for efficient long-term treatment of ocular diseases because they efficiently and stably transduce retinal pigment epithelium and photoreceptor cells following subretinal injection (93,252–257). Retinitis pigmentosa (RP) is a group of inherited retinal degenerative diseases that lead to progressive reduction in visual field extent and impairment of visual acuity. The disease is triggered by mutations in various genes that cause degeneration and death of photoreceptors by apoptotic pathways (258).

Modulation of photoreceptor apoptosis may offer an effective therapeutic approach to RP. AAV vectors carrying various genes including those encoding for cilliary neurotrophic factor; fibroblast growth factors FGF-2, FGF-5, and FGF-18; and glial cell line-derived neurotrophic factor were evaluated in rodent models of RP subretinal injections. In general, these

studies demonstrated long-term expression of the transgene, delayed photoreceptor degeneration, increased rod photoreceptor survival, and functional improvement (260–263).

Other therapeutic approaches to RP are aimed at specific mutant genes. Twelve percent of Americans with the blinding disease, autosomal retinitis pigmentosa, carry an autosomal dominant P23H mutation in their rhodopsin gene, and a similar transgenic rat model of this disease is available. Delivery with an AAV vector of a ribozyme targeted at this mutation in the rodent model, protected photoreceptors from death and resulted in significantly slowing the degenerative disease for 3 months (264). Mutations in the gene *Prph2*, which encodes the photoreceptor-specific membrane glycoprotein peripherin-2, cause several photoreceptor dystrophies, including autosomal dominant retinitis pigmentosa and macular dystrophy. A common feature of these diseases is the loss of photoreceptor function, also seen in the retinal degeneration slow ($Prph2^{Rd2/Rd2}$) mouse. Subretinal injection of AAV- Prph2 in these mice gave stable restoration of photoreceptor ultrastructure and electrophysiological function correction (265).

Control of angiogenesis in the retina is essential to the preservation of vision. Ocular neovascularization (NV) is a major threat to vision and a complicating feature of many eye diseases, including proliferative diabetic retinopathy, age-related macular degeneration, and retinopathy of prematurity. Regulation of vascularization in the mature retina involves a balance between endogenous positive growth factors (e.g., vascular endothelial growth factor), and inhibition of angiogenesis [e.g., pigment epithelium-derived factor (PEDF)]. Several studies examined ocular administration of AAV vectors to mice exhibiting ischemia-induced retinal NV. Expression of antiangiogenic proteins, either PEDF or the Kringle domains 1–3 of angiostatin (K1K3), gave sustained therapeutic levels of PEDF and K1K3 in the mouse eye and significantly reduced the level of retinal NV (266,267). Expression of a soluble VEGF receptor also led to significant reduction in the number of neovascular endothelial cells and inhibited retinal NV (268,269).

Recently, efficacy of AAV-based gene therapy directed at photoreceptors and retinal pigment epithelium has been demonstrated in a canine model of Leber congenital amaurosis, a childhood blindness disease. Intraocular administration of AAV-RPE65, encoding the gene for a 65-kD membrane-associated protein involved in retinoid metabolism to $RPE65^{-/-}$ dogs, resulted in partial restoration of visual function (270).

F. Rheumatoid Arthritis

Rheumatoid arthritis (RA), the most common inflammatory joint disease, is a chronic autoimmune disorder that affects approximately 1% of the population and causes significant disability. The etiology of RA is largely unknown, although current evidence suggests contributions from both environmental and genetic components (271). The chronic inflammation in the arthritic joint is characterized by recruitment of immune cells, including lymphocytes, macrophages, and plasma cells, leading to massive thickening of the synovium accompanied by release of inflammatory mediators, ultimately leading to invasion and destruction of articular cartilage and bone. At the molecular level, chronic inflammatory arthritis is characterized by diminution of T cell factors and an abundance of cytokines and growth factors, such as interleukin-6, tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β), which are produced by macrophages and synovial fibroblasts and play a major role in the progression of joint destruction (272). IL-1β, in particular, is a key cytokine that induces cartilage degradation, whereas TNF-α is a major cytokine involved in joint inflammation (273).

Conventional treatment to manage the symptoms of arthritis uses general anti-inflammatory agents, including both steroidal and nonsteroidal drugs, and disease-modifying drugs such as methotrexate. However, none of these pharmacologic agents have yet proven effective in halting the progression of disease. Recently introduced biological agents are more effective in ameliorating arthritis symptoms and halting the progression of disease. In particular, inhibitors of TNF-α and IL-1β have proven effective in preclinical studies as well as in human clinical trials, and the Food and Drug Administration (FDA) has approved the use of IL-1 receptor antagonist (IL-1Ra), soluble TNF-α receptor immunoglobulin Fc, and an anti-TNF-α monoclonal antibody for the treatment of RA.

The use of biologics for arthritis therapy presents several challenges. Because of the relatively short half-life of these proteins and the need for frequent, daily, or weekly dosing, effective levels of the therapeutic protein may not be maintained for extended periods. In addition, therapeutic proteins are administered systemically and may have reduced bioavailability in some affected joints. Also, production of biologics such as these proteins often is inefficient, leading to low yields and consequently high costs. Gene transfer may be a more efficient means of delivery of these biological agents. If persistent transgene expression could be achieved following gene transfer, it should circumvent the need for frequent repeat dosing and should allow attainment of steady levels of the product, as opposed to the peaks and troughs associated with intermittent protein administration. Furthermore, with the current advances in manufacturing, the production cost of vectors such as AAV may compare favorably with production costs of biologics.

AAV vectors encoding genes that selectively target key mediators, or that interfere with key biological processes, involved in the pathogenesis of RA have been evaluated in rodent models of inflammatory arthritis. In mice with collagen-induced arthritis (CIA), an AAV vector encoding interleukin-4 demonstrated protection, for up to 7 months, from articular cartilage destruction and amelioration of disease severity (274,275). The therapeutic efficiency of an AAV vector carrying the viral interleukin-10 (vIL-10) under the transcriptional control of the regulated TetON system was evaluated following intramuscular administration to mice with CIA. Expression of vIL-10, specifically induced by doxycycline, persisted for at least 4 months and reduced significantly the incidence and severity of arthritis (275a). In TNF-α transgenic mice, intra-articular injection of AAV encoding soluble TNF recep-

tor type I significantly decreased synovial hyperplasia, and cartilage and bone destruction (276).

In the streptococcal cell wall-induced rat arthritis model, administration of an AAV vector encoding the rat TNFR:Fc fusion gene, either systemically (intramuscular) or locally (intra-articular), resulted in profound suppression of arthritis. This was reflected in decreased inflammatory cell infiltration, pannus formation, cartilage and bone destruction, and mRNA expression of joint proinflammatory cytokines (277). Moreover, administration of the vector to one joint suppressed arthritis in the contralateral joint. An AAV vector encoding IL-1Ra cDNA was evaluated in a lipopolysaccharide (LPS)-induced arthritis model in rats using therapeutic, recurrence, and preventative protocols (278). IL-1Ra expression was upregulated by LPS-induced joint inflammation and proved efficacious in all protocols. Importantly, the IL-1Ra transgene persisted for more than 3 months and could be induced to express therapeutic levels of soluble IL-1Ra upon LPS administration. This resulted in suppression of inflammation and IL-1β production in the treated knee joints.

G. Lysosomal Storage Diseases

Lysosomal storage diseases (LSDs) are a large class of at least 40 rare genetic diseases that cause a deficiency in one of numerous acidic hydrolases that normally function in lysosomes to catabolize glycoproteins, glycolipids, or glycosaminoglycans [for a review, see (279)]. Most of these diseases are autosomal recessive, except for Hunter and Fabry, which are X linked. The enzyme deficiencies lead to accumulation of toxic substrates and can affect many organ systems. These proteins can be secreted and taken up by other organs via mannose-6-phosphate receptors, and this has allowed development of protein therapies for Gaucher and Fabry disease. However, most of these diseases also have a CNS pathology, which adds an additional challenge because systemically secreted proteins are not taken up across the blood–brain barrier. Consequently, gene therapy will likely require delivery both systemically, as well as directly to the brain, to correct the neurological defects. An additional challenge is that these diseases develop early in life; thus, the target patient population will likely be infants, even though therapy will be required for life. However, there are a large number of animal models and some impressive corrections of the disease in these models have been demonstrated with AAV vectors.

One well-characterized animal model is the rodent model of MPSVII, which is also know as Sly syndrome. The animal model shows many of the clinical and behavioral abnormalities of the human disease resulting from the deficiency of β-glucuronidase (GUSB) and the consequent accumulation of glycosaminoglycans. Intravenous administration (280) but not intramuscular injection (281) of an AAV-GUSB vector into MPS VII animals resulted in biochemical correction of the disease, although correction of the neurological and behavioral defects and cognitive function required direct injection into the brain (282–284). Injection of the vector into neonates in order to circumvent the blood–brain barrier resulted in prolonged correction of the disease in the GUSB-deficient mice for over 1 year (214). In this later study, as noted above, there was an anecdotal finding of some development of primarily hepatic tumors in some of the long-term corrected animals, but there was no evidence that these tumors were caused by the AAV vector (215,216)

Fabry disease results from a deficiency of the enzyme α-galactosidase A (α-galA) and is characterized by accumulation the lipids globotriaosylceramide (GL3) in the vascular endothelium, leading to CNS defects, vascular occlusion, and particularly renal dysfunction. An AAV2-α-galA vector delivered via the portal vein into Fabry mice showed enhanced levels of the enzyme and corresponding reductions in the levels of GL3 in liver, spleen, and heart for up to 6 months (285).

Pompe disease is caused by a lack of the enzyme acid α-1,4-glucosidase (GAA) and leads to excessive accumulation of glycogen and generalized myopathy of both skeletal and cardiac muscle. Treatment of $Gaa^{-/-}$ mice with an AAV2 vector containing the GAA cDNA by intramuscular or intramyocardial delivery resulted in near normal enzyme activities, and skeletal muscle contractility was restored as measured in the soleus muscle. Interestingly, intramuscular delivery of an AAV1 GAA vector lead to expression of the enzyme at more than 8 times the normal level with concomitant glycogen clearance (286).

H. Vaccines

Early reports on administration to animals of AAV vectors expressing some neoantigenic transgenes demonstrated the long-term durability of transgene expression in the muscle and showed that the use of AAV vectors did not result necessarily in an immune response against the vector-encoded transgene (54,55,287,288). However, other studies in mice showed that under certain conditions both humoral and antigen-specific T cell responses to the transgene protein could be observed. Production of these immune responses probably depends on several parameters that include the nature of the neoantigen and its level of expression and localization within transduced cells, the mouse strain used, and the vector dose. Transgene-specific immune responses have been observed in mice following administration of AAV vectors expressing genes encoding *Escherichia coli* β-galactosidase (203,289,290), ovalbumin (199), HSV-2 glycoproteins B (gB) and D (gD) (204), influenza virus hemagglutinin, and HIV-1 Env (290–292), human alpha-1-antitrypsin (97), and clotting factor IX (206).

The ability to elicit immune responses implies that there is no general mechanism by which AAV blunts the immune system. Although the mechanism of antigen presentation following administration of recombinant AAV vectors to the muscle is not clear, one report suggests that activation of T cells in the draining lymph nodes occurs exclusively through cross-presentation by antigen-presenting cells rather than by direct transduction of dendritic cells (DCs) (289). Whether cross-presentation is a phenomena occurring for all transgenes in the context of recombinant AAV vectors is unknown, but

it suggests a possible role for these vectors as vehicles for prophylactic or therapeutic vaccines. For example, one study demonstrated that a recombinant AAV vector expressing a secreted HSV-2 gB led to the activation of gB-specific CTL, which were most likely activated via cross-presentation of the secreted protein by DC (195). In contrast, mice injected intramuscularly with an AAV vector expressing ovalbumin developed a robust humoral response to the transgene product but only a minimal ovalbumin-specific CTL response (199).

The use of AAV to stimulate an antihuman immunodeficiency virus (HIV-1) response has been tested in mice. A single, intramuscular injection of an AAV vector encoding the HIV-1 *env*, *tat*, and *rev* genes (AAV-HIV) induced robust, long-term production of HIV-1-specific serum IgG and MHC class I-restricted CTL activity (292). HIV-specific cell-mediated immunity was enhanced strongly by coadministration with an AAV vector encoding interleukin-12, whereas boosting with AAV-HIV resulted in rapid and strong HIV-1-specific humoral responses. When AAV-HIV was administered orally, a strong systemic and regional HIV-specific humoral immunity and MHC class I-restricted CTL response was induced, which significantly reduced viral load after intrarectal challenge with a recombinant vaccinia virus expressing the HIV-1 *env* gene (293).

In rhesus macaques, a single-dose, intramuscular administration of an AAV vector expressing the simian immunodeficiency virus (SIV) major structural genes resulted in long-term $CD8^+$, antigen-specific CTL responses against multiple SIV protein epitopes that were similar to responses observed in monkeys directly infected with pathogenic SIV. Neutralizing antibody responses were also robust and persisted for more than 1 year. More recent studies have begun to test the efficacy of AAV-SIV vaccines in an SIV-macaque challenge model. The data so far strongly suggest that immunized macaques have significantly lower virus burden at peak (2 weeks) and set point (10 weeks) after intravenous challenge with SIV than do mock-vaccinated control animals (294).

AAV vectors are also being examined as potential vehicles for tumor vaccines. In one study, an AAV-based tumor vaccine was developed by constructing a chimeric gene containing a human papillomavirus (HPV) type 16 E7 CTL epitope fused to a heat shock protein. When administered intramuscularly, this vaccine was able to eliminate tumor cells in syngeneic animals in a manner that was dependent on $CD4^+$ and $CD8^+$ cells. This suggests that vaccination with this gene could be a therapeutic treatment for cervical cancer containing HPV-16 E7 (295)

XI. SUMMARY

The work summarized in this chapter illustrates the increasingly rapid progress that is now being made in developing therapeutic applications of AAV vectors. The early clinical testing of AAV vectors for the treatment of CF has been extremely important in initiating the regulatory environment for AAV vectors and demonstrating the inherent good safety profile of AAV vectors. As more groups have extended investigations to additional in vivo models, the potential utility of AAV vectors as therapeutic gene delivery vehicles has gained more widespread interest. The development of more sophisticated production systems for AAV vectors has enhanced both the quantity and quality of vectors that can be produced. Additional work to modify the transduction efficiency by judicious choice of serotype for the capsid or by modification of the capsid structure will expand the use and potency of AAV vectors. In addition, further understanding of the intracellular metabolism of AAV vectors also will increase the sophistication with which these vectors can be deployed. It is likely that in the next few years there will be a significant increase in clinical testing of AAV vectors for additional therapeutic applications.

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6

Engineering Herpes Simplex Viral Vectors for Therapeutic Gene Transfer

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I. INTRODUCTION

A. General Overview

Research into the genetic foundations of disease, both acquired and inherited, has fostered progress of molecular medicines. Exemplified by the recent decoding of the complete human and mouse genomes; medical scientists have ever-expanding tools useful to engineer and employ therapeutic gene transfer in medical research programs. In contrast to traditional drugs, which act by modifying existing gene product activity, therapeutic gene transfer, or gene therapy, alters the genetic makeup of the cell with the intention of ameliorating the target disease phenotype. Successful gene therapy requires the identification of therapeutic genes that will correct a genetic defect or rectify a disease process, as well as the design and construction of suitable vehicles for delivery and expression of these genes in vivo. Identification of genes with therapeutic potential and engineering of effective gene transfer vectors are rapidly proceeding, making gene therapy an imminent practical reality. Considerable progress has been made in vector design, and clinical trials for the treatment of a number of diseases are underway. Success in these initial gene therapy endeavors is providing incentive for research into additional gene therapy applications, holding promise for creating a new age of molecular medicine in which genomics and proteomics dovetail with genetic diagnostics and therapeutic gene transfer.

Although remarkable progress has been made in the development of gene vector technologies, substantial hurdles remain. These include the development of strategies for vector targeting, modifications to increase transgene stability, regulation of gene expression, and circumvention of undesirable immune responses. Delivery of transgenes into target cells can be accomplished using either viral or nonviral vectors, with viral strategies remaining the most prevalent in human clinical trials. Virus-mediated gene delivery requires efficient methods for vector construction, vector production, and target cell infection. In this chapter, the relative merits and potential applications of herpes simplex virus type 1 (HSV-1) vectors are discussed. In the context of vector development, the natural history of HSV infection in the host is reviewed, highlighting unique features of the virus biology. Remaining potential pitfalls and approaches to their solution are also discussed.

B. Advantages of HSV Vectors

Although each viral gene transfer vector possesses distinct attributes, successful gene therapy will require construction of gene transfer vectors that are tailored to specific applications. The human herpes viruses represent promising candidate vectors for several types of gene therapy applications that include neuropathological disorders, cancer, pain control, autoimmune syndromes, and metabolic diseases.

Herpes viruses are large DNA viruses, with the potential to accommodate multiple transgene cassettes, that have evolved mechanisms that allow lifelong persistence in a nonintegrated latent state without causing disease in an immune-competent host. Among the herpes viruses, HSV-1 is an attractive vehicle for gene transfer to the nervous system because natural infection of humans results in a usually benign, lifelong persistence of viral genomes in neurons. This latent state is characterized by the absence of lytic viral protein expression, and the pres-

ence of these latent genomes does not alter nerve cell function or survival. The HSV-1 genome contains a unique, neuron-specific promoter complex that remains active during latency, the latency active promoter (LAP). This promoter can be adapted to express therapeutic proteins without compromising the latent state or stimulating immune rejection of transduced cells. The establishment of latency does not appear to require the expression of viral lytic functions. Essential genes required for expression of the viral lytic functions can therefore be deleted to create completely replication-defective vectors that nonetheless effectively establish a latent state, but cannot cause disease or reactivate from latency. Experimental HSV infection is not limited to neurons; the virus is capable of infecting most mammalian cell types, and does not require cell division for infection and gene expression. Accordingly, HSV may be generally useful for gene transfer to a variety of nonneuronal tissues, particularly where short-term transgene expression is required to achieve a therapeutic effect.

Considerable technical progress has been made in developing HSV-1 into a practical gene transfer vector, including the development of efficient methods for vector construction and high-titer vector production. The obstacles requiring satisfactory resolution in order to realize the full potential of these vectors include (1) the elimination of vector toxicity, (2) the design of promoter cassettes that provide for sufficient level and duration of transgene expression, and (3) targeting of transgene expression to specific cell populations through the use of tissue-specific promoters, or by altering the virus host range through modifying receptor utilization for attachment and entry. This chapter concentrates on the design, production, and utilization of replication-deficient genomic HSV vectors.

II. VECTOR DESIGN STRATEGIES

A. Biology of the Viral Lytic Cycle

HSV-1 is a double-stranded DNA virus whose capsid is surrounded by a dense layer of proteins, the tegument, which is contained within a lipid bilayer envelope (Fig. 1A). Glycoproteins embedded in the viral envelope mediate infection of the host cell, which takes place in 2 identifiable stages: (1) attachment to the cell surface, and (2) fusion with the cell membrane, resulting in virus penetration. The envelope of HSV-1 contains at least 10 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gL, and gM) and 4 nonglycosylated integral membrane proteins (products of the UL20, UL34, UL45, and UL49.5 genes). Of the 10 glycoproteins, gB, gD, gH, and gL are essential for viral infection (1–4), whereas gC, gE, gG, gI, gJ, and gM are dispensable for infection in vitro (5–7).

Attachment of the viral particle is mediated by several glycoproteins (5,6,8). The sequential attachment steps in infection result in fusion of the viral envelope with the cell surface membrane and entry of the viral capsid into the cell cytoplasm. Even though the molecular events of penetration are not well understood, it is clear that multiple viral glycoproteins are required (e.g., gB, gD, gH/gL) (2,4,9–11). In addition, following new virion assembly, viral glycoproteins are also involved in a less well-defined process of egress and release of mature particles from the infected cell membrane. Viral particles are also capable of spreading from cell to cell across cell junctions, a process requiring the functional activities of several glycoproteins that are not required for initial infection (e.g., gI/gE) (12,13).

The genome structure of HSV can be divided into viral genes that are essential or accessory for replication in cell culture (Fig. 1B). The accessory functions may be deleted without significantly hampering virus growth in culture. However, removal of essential genes necessitates the use of complementing cell lines that express the essential products in order to propagate these viral recombinants. In human infections, HSV binds to and enters epidermal cells following direct contact with an infected individual that is shedding virus or has an active lesion. Following virus attachment, the viral capsid penetrates the surface membranes of epithelial cells of the skin or mucosa and is transported to the nuclear membrane where viral DNA is injected through a nuclear pore (Fig. 2A). Once inside the nucleus, the viral DNA is circularized and transported to nuclear domain 10 (ND10) structures (14,15), where the immediate early (IE) genes are expressed as part of the sequential cascade of lytic gene synthesis (16) (Fig. 2B). Transcription of the 5 IE genes (ICP0, ICP4, ICP22, ICP27, and ICP47) does not require *de novo* viral protein synthesis. Expression of the IE genes is controlled by promoters that contain 1 or more copies of an enhancer element responsive to the viral tegument protein VP16 (a.k.a. Vmw 65 or TIF, a transactivator that is transported into the nucleus along with viral DNA) (17–19). The IE genes ICP4 and ICP27 encode products required for expression of the early (E) and late (L) genes (20–23); the former (E) gene class specifying primarily enzyme functions required for viral DNA synthesis and the latter (L) comprising primarily virion structural components. ICP4 regulates viral promoter function (21), whereas ICP27 affects the processing and transport of viral RNA (24,25). The IE gene products ICP0 and ICP22 contribute to viral gene transcription but are not essential to virus replication in cultured cells (26–29). ICP0 is a promiscuous transactivator that exerts its effect prior to the transcription initiation event; it is not a DNA-binding protein (30). ICP22 has been found to regulate the level of ICP0 expression (31). ICP47 does not affect transcription but rather has been reported to interfere with a transporter function transporter of antigen presentation (TAP) that is responsible for loading major histocompatibility (MHC) class I molecules with antigenic peptides (32–35). Expression of late genes is dependent on both viral DNA synthesis and IE gene functions (21,23,36,37). Following translation of the late gene products, which become viral structural components of the capsid, tegument, and envelope, genome-length copies of viral DNA are packaged into the newly assembled capsids. Tegument proteins accumulate around the capsid and the immature particle buds through the inner nuclear membrane where the viral glycoproteins are localized. Double-membrane-enveloped virus containing virus-encoded glycoproteins modified by the Golgi apparatus

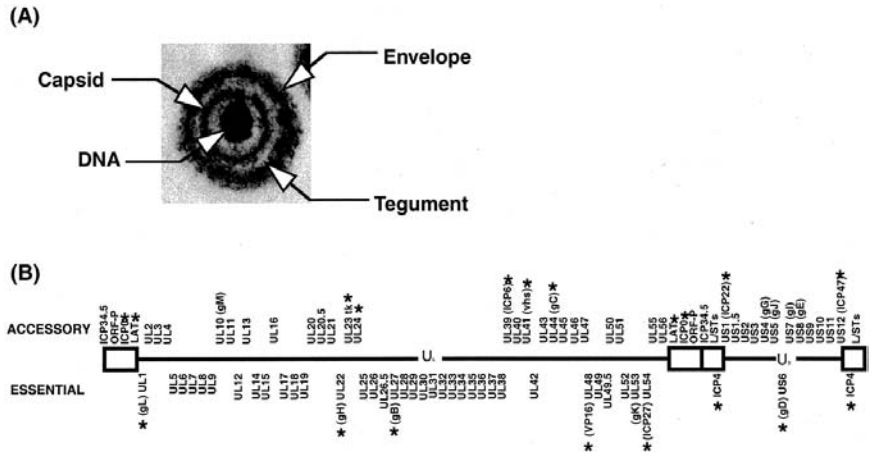

Figure 1 HSV-1 virion structure and genome organization. (A) Electron micrograph of the HSV particle, showing the capsid, tegument, and glycoprotein-containing lipid envelope. (B) Schematic representation of the HSV genome, showing the unique long (U_L) and unique short (U_S) segments, each bounded by inverted repeat (IR) elements. The location of the essential genes, which are required for viral replication in vitro, and the nonessential or accessory genes, which may be deleted without affecting replication in vitro, are indicated. The 5 IE genes, various glycoprotein genes, LAT, and other important loci are highlighted by asterisks.

enzymes fuse with the cell membrane forming a mature, extracellular virus particle with a single membrane bilayer (38). The infectious particles can infect neighboring cells by cell-to-cell transmission or can be released for infection of distal cells. With the exception of sensory neurons, cell lysis accompanies productive viral infection.

B. Complementation of Essential Genes and Elimination of Cytotoxicity

Because HSV genes are expressed in a sequential cascade during lytic infection (16), removal of the single essential IE gene ICP4 severely inhibits the expression of later E and L genes (21), resulting in a defective vector incapable of producing virus particles. In addition, the IE gene products, with the exception of ICP47, are individually toxic to most cell types when expressed at high levels by transfection (39). The elimination of multiple IE genes reduces the cytotoxicity of HSV-based vectors for cell lines and primary neuronal cell cultures (40–44) (Figs. 3A and B). Cytotoxicity of a genomic HSV vector deleted for ICP4, ICP22, and ICP27 in cultured Vero cells is reduced compared with a virus-only deficient for ICP4 (44). Such mutants are also less toxic to primary neurons (41) or undifferentiated cells such as umbilical cord blood-derived stem cells (Wechuck and Glorioso, unpublished observation, 2003). Mutants deleted for ICP0, ICP4 and ICP27 and defective for ICP22 expression in noncomplimenting cells are essentially nontoxic, even when infected at very high multiplicities (45); in this regard they are similar to UV-irradiated particles that do not express viral functions (46). We created a mutant background for gene therapy applications that require short-term, high-level expression by constructing an HSV-1 vector deleted for multiple viral functions, including the IE genes ICP4, ICP22, ICP27, and ICP47. We chose to remove the ICP47 gene in this vector to avoid interference with antigen presentation in applications intended for induction of specific immunity, but we did not delete the ICP0 gene because this gene product improves transgene expression and permits efficient construction of recombinant vectors (47,48). In this mutant background, we eliminated the virion host shut-off function (vhs) encoded by UL41 because this virus tegument component indiscriminately interferes with translation of mRNA in infected cells (49–51). A similar vector background has been used to express transgenes for up to 21 days in cultured primary neurons without causing neuronal cell death (41). For applications involving infection of cancer cells in vivo, the transient arrest of cell division offered by ICP0 and subsequent recovery of cell growth at high multiplicity of infection should prove advantageous because transducted cells produce high levels of transgene product prior to induction of cell death.

C. Vector Transgene Capacity

The treatment of monogenic diseases requires only limited vector capacity, but complex applications may require the delivery of large or multiple independent genetic sequences. A comparison of the genome structure and capacity of several current vector systems is shown in Fig. 4. The overall size of the HSV-1 genome (152 kb) represents an attractive feature for employing the vector for the transfer of large amounts of exogenous genetic sequences. Approximately one-half of the HSV-1 coding sequences are nonessential for virus replication in cell culture and therefore may be deleted to increase transgene capacity without blocking viral replication (Fig.

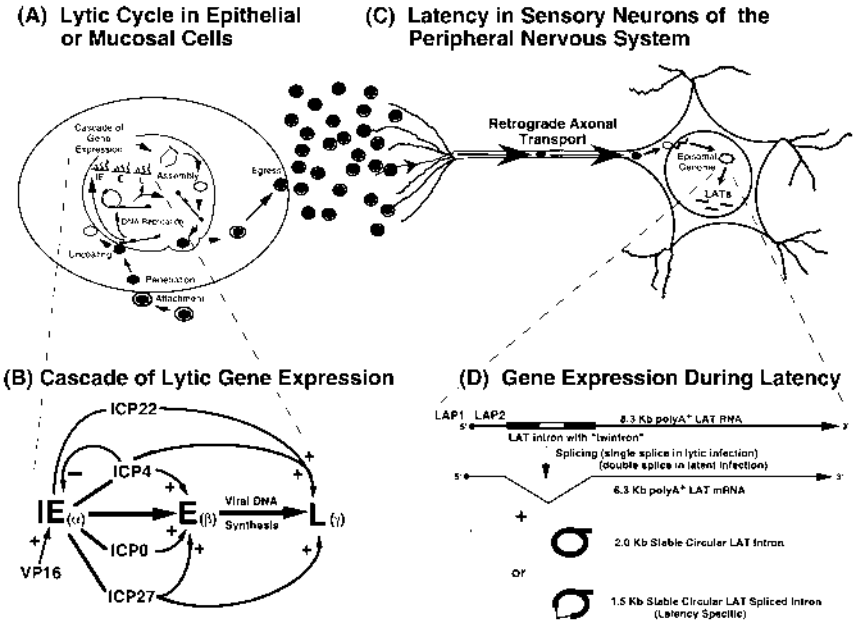

Figure 2 HSV-1 life cycle in the host. (A) Lytic infection. Primary lytic infection of epithelial or mucosal cells results from the attachment and penetration of HSV particles to host cells, a complex process involving many HSV surface glycoproteins. Following transport of the capsid to the nuclear membrane and injection of linear dsDNA into the nucleus, the genome circularizes and begins to express the lytic HSV gene functions in a highly regulated sequential cascade, yielding the expression of proteins involved in viral DNA synthesis and virion structural components. Following assembly of newly synthesized particles within the nucleus, virion maturation results in the egress of these virions from the infected cell. (B) Schematic diagram of the sequential cascade of lytic gene expression. The 5 IE or α genes are expressed immediately upon infection through transactivation by the VP16 tegument protein. The ICP4, ICP27, and ICP0 IE gene functions are responsible for the activation of the early or β genes that are primarily involved in viral DNA synthesis. In addition, ICP4 acts to shut off expression of the IE genes. Following viral DNA replication, ICP4, ICP22, and ICP27 participate in the activation of true viral late or γ genes, which mainly encode virion structural components. (C) Latent infection. When virion particles encounter and bind to axonal termini that innervate the site of primary infection, viral capsids are transported in a retrograde manner to the nerve cell body. At this point the circular viral genome can persist as an episomal molecule in a latent state within the neuron, wherein viral lytic gene expression is silenced and a series of latency-associated transcripts (LATs) are produced. (D) Gene expression during latency. The major 2.0-Kb latency-associated transcript (LAT) arises from the large 8.3-Kb polyA$^+$ through a splicing event that yields an unstable 6.3-Kb LAT and a circular LAT lariat of 2.0 Kb. The location of the latency active promoter (LAP) regions LAP1 and LAP2 relative to the LATs is depicted.

1B). The latency region of the virus genome represents approximately 8 kb of sequence that can be removed and the joint region of the virus is composed of 15 kb of redundant sequence that can be eliminated without compromising virus replication (52). In one set of experiments we removed an 11-kb section of the U_S region of the genome (Laquerre and Glorioso, unpublished data, 1998) containing gD, the only essential gene in this region, which can be propagated on a cell line that expresses gD in trans (4). Approximately 44 kb of HSV sequence can potentially be removed and vectors propagated in cells engineered to complement just 3 viral functions (ICP4, ICP27, and gD). Transgene expression cassettes can also be inserted into deleted essential gene loci to avoid transfer of foreign sequences to wild-type virus by recombination that could potentially occur between the vector and wild-type genomes in vivo. We have observed that some nonessential genes (e.g., IE genes ICP0 and ICP22) are toxic to some cell types, yet the products of these genes are required for high-titer vector production. The toxicity of these products makes it difficult to produce a complementing cell line carrying these genes. However, it is possible to engineer the promoters for these genes in a manner to make their function dependent on viral IE genes present only in complementing cells (44). By the judicious selection of viral gene deletions and promoter alterations, high-titer vectors can be produced with minimal complementation.

We have developed a panel of novel HSV-1 vectors with a background suitable for expression of multiple transgenes

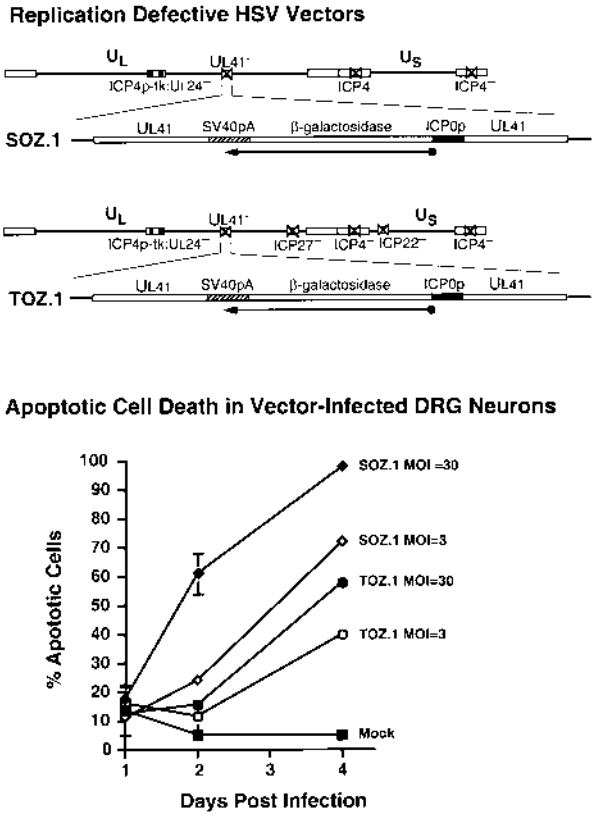

Figure 3 Reduced toxicity following infection of primary dorsal root ganglia neurons. (A) Schematic representation of the first-generation SOZ.1 (ICP4$^-$, UL41$^-$:ICP0p-lacZ: UL24$^-$:ICP4p-tk) and the third-generation TOZ.1 (ICP4$^-$, ICP22$^-$, ICP27$^-$, UL41$^-$:ICP0p-lacZ, UL24-:ICP4p-tk) replication-defective vectors displaying the lacZ transgene inserted into the UL41 locus under the control of the ICP0 promoter using the SV40 polyadenylation signal. (B) The number of primary dorsal root ganglion (DRG) neurons undergoing apoptosis was determined at various times following infection with either SOZ.1 or TOZ.1. Even at an MOI of 30, TOZ.1 was less toxic than the first-generation vector (SOZ.1) at the lower MOI (3.0).

using a rapid gene insertion procedure (53). To take advantage of the reduced cytotoxicity resulting from the deletion of ICP4, ICP22, and ICP27 genes (41,43), we designed a single vector in which 9 viral genes were deleted, removing a total of 11.6 kb of viral DNA that was replaced with multiple transgenes under control of different promoters. These HSV multigene vectors were constructed with either 4 or 5 independent transgenes at distinct loci (47) with all the transgenes simultaneously expressed for up to 7 days. These multigene vectors demonstrate the potential for using HSV-1 vectors for the expression of complex sets of transgenes that have coordinated or complementary functions.

Recent work inserting bacterial artificial chromosome (BAC) elements into large viruses including HSV promises to increase the speed of recombinant construction as well as allow the creation of extensively modified vectors without the necessity of creating complementing cell lines. We have introduced the BAC elements into multiply defective HSV vectors and propagated them in *Escherichia coli* (Wolfe and Glorioso, unpublished data, 2003). Transfection of HSV:BAC DNA purified from bacteria into complementing cells is much more efficient than using viral DNA purified from mammalian cells. Infectious virus produced can be recovered back into *E. coli* for further modification. Several methods have been used to modify the viral genome in bacteria with high frequencies of recombinant production in a greatly shortened time frame. We are further modifying vector genomes and engineering additional methods for recombinant engineering in bacteria.

D. Amplicons as an Alternative Vector System

Often referred to as ''defective'' HSV-1 vectors, amplicons are plasmids engineered to contain both an HSV origin of replication and packaging signals and a bacterial origin of replication (54). Amplicons are propagated in bacteria and then cotransfected with a defective HSV ''helper'' virus to create a mixed population of HSV particles containing either the defective HSV helper genome or concatemers of the plasmid packaged within an HSV capsid. In concept, the production of virion-packaged amplicons uses transient complementation of the entire HSV genome to provide replication machinery and viral structural components. A comparison of replication-defective genomic HSV vectors and helper virus-free amplicons are shown in Figs. 5A and B). Amplicons have been used to express reporter genes (55–61) or biologically active peptides (55,62–70) transiently in tissue culture systems.

In vivo, prolonged expression of both a *lacZ* reporter gene (59,71–75) and of the TH gene (71) following amplicon injection into brain have been reported. However, the production of amplicons requires repeated passaging of the amplicon/helper virus preparation, which results in the emergence of recombinant wild-type virus that, although estimated to occur at the low frequency of 10^{-5} (71,72), results in the death of 10% of infected animals in experiments in vivo (71). The production of true helper virus-free amplicon preparations using multiple restriction fragments of the helper virus genome that lack packaging signals has recently been reported (76). However, the maximal yield obtained with that method has remained low ($<10^7$ pfu/mL), and expression in vivo has not been fully tested (76). The presence of cytotoxic helper virus and the generation of replication-competent contaminants represent technical hurdles to the effective production and use of amplicons in human patients. Helper-free amplicons will likely require the development of new helper systems to make their use practical enough for human applications.

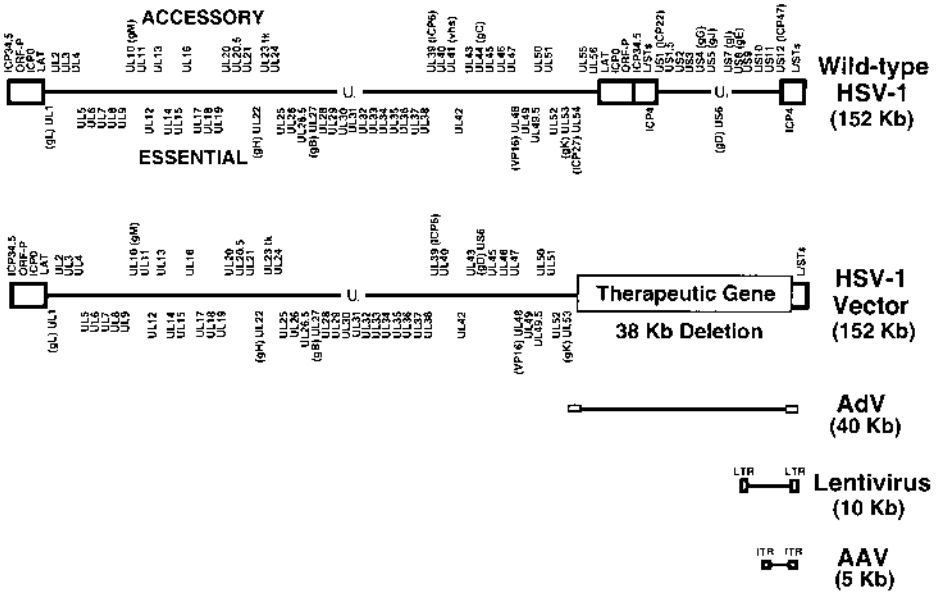

Figure 4 Comparison of viral vector payload capacities. Schematic diagram of various viral vector genomes currently in use for gene transfer and therapy studies, including the overall size of the entire vector genome. The HSV-1 vector, which contains a 38-kb deletion of sequences comprising the joint region and the entire unique short (U_S) segment of the viral genome (ICP4$^-$, ICP22$^-$, ICP27$^-$), can accommodate foreign trangene sequenes that are larger than lentivirus or AAV vectors and equivalent in size to the complete adenoviral (AdV) genome.

III. VECTOR TARGETING

One method to achieve cell-specific expression of the therapeutic gene is targeting vector recognition and infection of cells to unique receptors present principally on target cells. Exploration of this approach has been limited thus far, but several recent reports have shown convincing targeting of adenovirus (77–79) or retrovirus (80–83). Targeted viral infection requires (1) the identification of cell-specific surface receptor(s) to which viral binding/entry can be directed, and (2) the modification of viral glycoproteins to recognize novel receptors while eliminating the binding of these viral ligands to native receptors, a process that ideally should be accomplished without compromising infectivity. These steps can be combined if it is possible to replace the natural receptor-binding domain of viral glycoproteins responsible for infection with binding domains specific for alternate receptors.

The complexity of the glycoproteins in the viral membrane and the fact that multiple glycoproteins are required for the sequential steps of virus attachment and entry has made redirecting HSV infection difficult. Early electron microscopic studies showed that the trans-plasmalemmal entry of HSV into cells occurs in two morphologically definable stages: cell surface attachment and virion–cell fusion. It is convenient to consider these stages separately, although in reality the transition from one to the other is rapid and seamless.

Initial virus attachment is cooperatively mediated by numerous glycoproteins (5,6,8). Binding of viral particles to cell surface heparan sulfate (HS) and other glycosaminoglycans (GAGs) (10,84–90) is mediated by exposed domains of glycoproteins C (91–94) and B (86,92), as evidenced by the diminished cell attachment of gC and gB mutants, and the interference with cell attachment by (1) pretreatment with anti-gB or anti-gC antibodies, (2) enzymatic removal of cell surface GAGs, and (3) competition studies using heparin. The role of gB is complex as this glycoprotein functions both in the initial binding of virus to the cell surface (nonessential for cell entry) and in later stages in the virion–cell fusion program (essential for cell entry). Molecular dissection of the domains of gB responsible for each distinct process has allowed the generation of a gB mutant that shows isolated loss of GAG-binding functions, dissociated from preserved cell entry functions. This mutant, generated by deletion of a positively charged lysine-rich region within gB (designated gB:pK$^-$), is an important resource in the generation of viral vectors with targeted cell binding and preserved entry (see below). The initial gB/gC-HS/GAG-mediated cell attachment greatly enhances but is not essential for subsequent events in the cell entry cascade; thus, cell attachment represents a reasonable target for strategies aimed at effecting restricted cell entry.

In initial studies to manipulate the cell attachment properties of HSV, we focused on the elimination of HS binding by removal of appropriate binding domains from the virus envelope, leaving intact the viral determinants mediating the downstream events in cell entry. We hypothesized that subsequent replacement of the HS-binding ligands with avid spe-

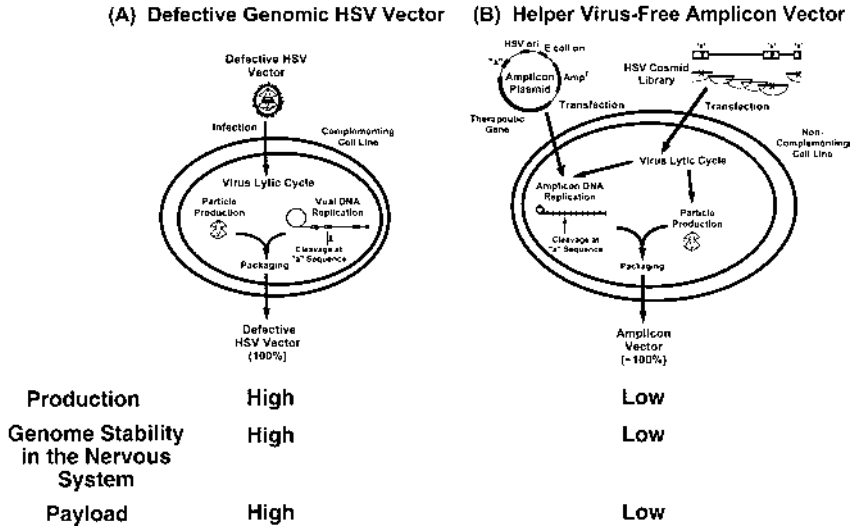

Figure 5 Strategies for HSV-1 vector design. (A) The production of defective full-length genomic HSV vectors is carried out in cell lines engineered to provide the deleted essential genes *in trans*. These vectors can be produced in high titers, are capable of long-term persistence in neurons in vivo, can accommodate large or multiple transgenes, and are incapable of replicating in neurons or other cells because of the missing essential genes. (B) Helper-free amplicons can be readily propagated in bacteria using the bacterial origin of replication (*E. coli* ori), and then transfected into a noncomplementing cell line along with 5 cosmids that encompass the entire HSV genome. Unlike the standard amplicon system in which the final preparation consists either of a mixture of amplicon concatemers and defective HSV particles, only amplicon concatemers get packaged into new virus particles because the overlapping cosmids lack the HSV packaging sequence (''a'' sequence). The helper-free amplicon preparations suffer from low titer yields, decreased stability of the amplicon DNA, and decreased transgene payload.

cific receptor-binding sequences might achieve at least partial targeting. A double-mutant virus, KgBpK-gC-, was derived from wild-type KOS strain (Figs. 6A and B). In this mutant, the coding sequence for the nonessential gC gene was removed from the viral genome and the wild-type gB sequence replaced by the gB:pK$^-$ mutation (92) (Fig. 6B). The resulting virus demonstrated an 80% reduction in binding to Vero cells compared with wild-type virus (Fig. 6D). By replacing the HS-binding domain of gC with the coding sequence of erythropoietin (EPO) in the background of the KgBpK$^-$gC$^-$ mutant virus (Fig. 6C), we demonstrated that the gC:EPO fusion protein was incorporated into the budding virion and that recombinant virus was specifically retained on a soluble EPO-receptor column (95). The gC:EPO virus demonstrated a 2-fold increase in infection of K562 cells (Fig. 6D) (Laquerre and Glorioso, unpublished data, 1998), which express the EPO receptor. The EPO-expressing particle also stimulated the proliferation of FD-EPO cells (95), an EPO-dependent cell line, indicating that virus binding to specific cellular receptor had occurred. However, virus attachment was followed by delivery of the viral particle to the endosome compartment resulting in virus degradation, thus preventing the normal pathway of entry resulting in productive infection. These studies stress the importance of appropriate receptor interactions to ensure virus entry by the normal route of virus penetration.

An alternative strategy for effecting targeted cell entry through engineering cell attachment was subsequently considered. In the gC-EPO experiments described above, entry of HSV into the endosome prevented cytoplasmic penetration, probably because of the low pH of the endosome compartment; trans-plasmalemmal cellular entry of HSV occurs at physiological pH, and it is unlikely that the native viral entry determinants would be functional in the acidic environment of the endosome. Several viruses, however, enter cells through a trans-endosomal route, exploiting the low pH of the endosome to effect fusion-triggering alterations in viral receptors. An example is provided by vesicular stomatitis virus (VSV). The VSV-G spike glycoprotein had previously been successfully used to redirect the tropism of lentiviruses, expanding their host cell range and reconfiguring the usual trans-plasmalemmal route of entry of lentiviruses into cells, to enable a trans-endosomal route to be taken. We thus explored pseudotyping HSV using VSV/HSV fusion proteins. A mutant virus was used in which the short unique segment of the HSV genome U_S3–8 (encoding gD in addition to nonessential glycoproteins gE, gG, gJ, and gI) was deleted. The gD-null phenotype of this vector was transiently rescued by using a series of plasmid expression cassettes encoding chimeric VSV-G/HSV glycoprotein fusion proteins (96). Chimeras containing the ectodomain of VSV-G linked to either the C-terminal or

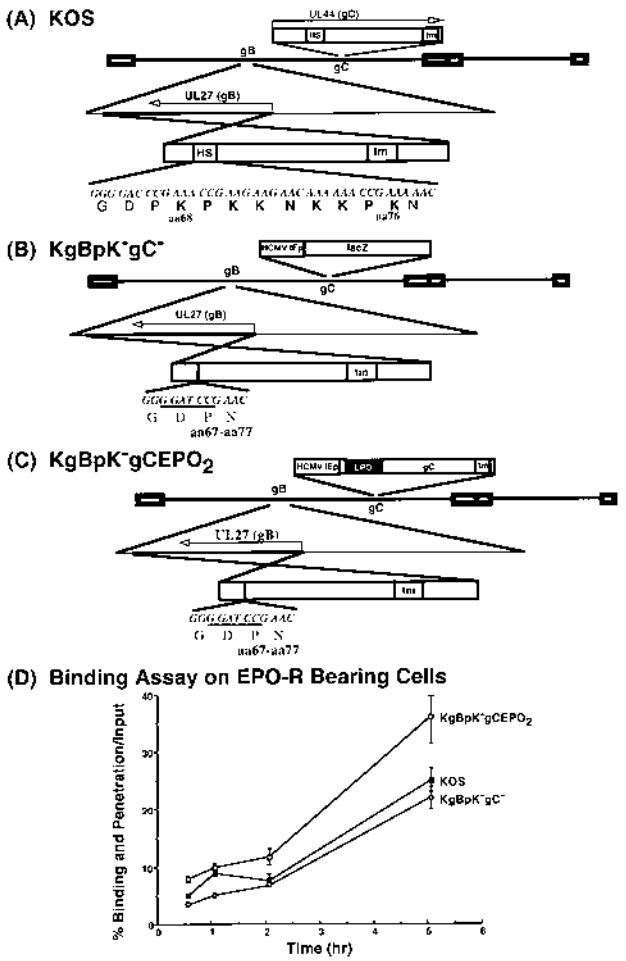

Figure 6 Targeted binding of HSV-1 particles expressing gC:EPO fusion molecules to EPO receptor (EPO-R)-bearing cells. (A) Diagram of the KOS wild-type HSV-1 genome depicting the location of the two HSV glycoproteins (gB and gC) involved in heperan sulfate-binding. The heperan sulfate binding domain of gB consisting of a series of polylysine (pK) residues is shown in greater detail. (B) The $KgBpK^{-}gC^{-}$ recombinant virus deleted for binding to heperan sulfate was constructed by deleting the pK region from the essential gB gene and deletion of the nonessential gC gene by insertion of an HCMV IEp-lacZ expression cassette into the gC locus. (C) The $gC{:}EPO_2$ recombinant ($KgBpK^{-}gCEPO_2$) was constructed by introducing EPO into the gC gene, replacing aa#1–162 in the $KgBpK^{-}gC^{-}$ recombinant virus, which could readily be purified by X-gal staining. (D) The percentage of radiolabeled wild-type HSV-1 (KOS), the recombinant deleted for heperan sulfate binding ($KgBpK^{-}gC^{-}$), and the EPO-expressing ($KgBpK^{-}gCEPO_2$) viruses that bound to K566 cells bearing the EPO-R was determined and is expressed compared with input virus. These data demonstrate that the $KgBpK^{-}gCEPO_2$ recombinant virus binds to the EPO-R and this binding conferred increased infectivity of the $KgBpK^{-}$gCEPO2 virus for cells bearing the EPO-R (K566).

transmembrane domain of either gD or gB were generated. A number of different constructs were tested to facilitate the identification of chimaeras that were effectively packaged into the virion, because it was previously suggested that the structural requirements for incorporation of glycoproteins into the envelope of the mature virus particle were stringent with respect to transmembrane or endodomains (97–100). It was demonstrated that VSV-G chimeras containing the transmembrane domain of gD, or a truncated gB transmembrane domain, were incorporated into the viral envelope efficiently, and that the wild-type VSV-G protein was incorporated rather less efficiently (96). The latter, however, was able to partially rescue the gD-deficient phenotype, whereas the chimeric proteins were nonfunctional. Neutralizing anti-VSV-G antibodies blocked the partial VSV-G mediated rescue (96). The poor efficiency of phenotypic rescue of the U_S3–8 null virus by VSV-G may be attributable to either (1) inefficient incorporation of the foreign viral glycoprotein, or (2) acid degradation of HSV in the endosome compartment. We have studied this issue further in order to elucidate and exploit the mechanisms contributing to partial rescue. A recombinant virus was generated in which the VSV-G expression cassette was incorporated into the genome of the U_S3–8 deleted virus (the resulting vector is null for gD, gE, gG, gJ, and gI, but expresses VSV-G). The recombinant particle enters cells possessing the VSV receptor. However, an abortive infection ensues, culminating in endosomal degradation of the virion at low pH, similar to that observed when the gC-EPO-expressing recombinant enters cells (Goins and Glorioso, unpublished data, 2003). The use of lysomotropic agents that raise endosomal pH, such as chloroquine (101), enables release of viral contents from the endosome into the cytoplasm, resulting in plaque formation. An acidic environment is required for the activation of VSV-G fusion functions (102,103). It follows that, in the presence of lysomotropic drugs, fusion of the VSV-G expressing, U_S3–8 null, recombinant with the endosomal membrane must be mediated by HSV fusion glycoproteins (gB or gH/gL). It thus appears likely that, in this situation, VSV-G functions as a ligand for an internalized receptor, rather than a pH-dependent mediator of viral envelope fusion with the endosome membrane. We are currently examining the mechanism in more detail because this strategy might be exploited to allow specific targeting of HSV to internalized cellular receptors.

Viral gC/gB-mediated binding to cell surface HS is followed by a second binding event between viral gD and specific cellular receptors. An essential role for gD in virus penetration is supported by the finding that recombinant virus deleted for gD is capable of binding to cells but is unable to penetrate (9,11). In addition, infection can be neutralized by anti-gD antibodies that do not block attachment of virus to the cell (9,11). Several distinct cellular gD receptors have been identified. The first HSV gD receptor herpesvirus entry mediator (HVEM or HveA), isolated by screening a cDNA expression library in HSV refractory Chinese hamster ovary (CHO) cells, was determined to be a member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family (104). Subsequently, a number of other entry mediators have been identi-

fied, including HveB [poliovirus receptor-related protein 2 (Prr2)] (105), and HveC [nectin-1, poliovirus receptor-related protein 1 (Prr-1)] (106). The latter, an alternatively spliced member of the immunoglobulin superfamily that bears no structural relation to HVEM/HveA is the major gD receptor and is widely expressed. Finding multiple, unrelated receptors capable of mediating HSV infection via gD suggests that several distinct receptor-binding domains exist within gD. The recent publication of the crystal structure of the HveA-gD receptor–ligand complex is an essential first step in the recognition of gD domains that are essential for interaction with specific cellular ligands. This knowledge may assume great importance in the rational design of gD mutants that are restricted for binding/entry functions through prespecified cellular HSV receptors, and ultimately for engineering gD to target new ligands. Targeted infection might be accomplished by manipulation of the specificity of gD while leaving the HS-binding activity of gC and gB intact. The ability of gD to bind to a range of cellular receptors suggests that the partial or complete substitution of gD with other sequences capable of mediating viral entry may provide a means of HSV vector targeting. Mutation of, or antibody binding to, N-terminal amino acids of gD eliminates HveA binding while leaving HveC binding and the penetration function intact, but the reverse has yet to be accomplished (107). Initial mutagenesis studies suggest that the binding and entry functions of gD may be dissociable and dependent on overlapping but distinct subsets of amino acid residues within the HveA/HVEM- and HveC/nectin-1/Prr-1-binding domains of gD (Bai and Glorioso, unpublished data, 2002), suggesting that targeted entry may be at least theoretically possible. A subsequent study has shown limited evidence that cell entry might occur through engineered gD (108). A recombinant virus was generated, in which the binding sites for HS in gB and gC were deleted. The amino-terminal of gC was replaced by IL-13 and a second copy of IL-13 was inserted into gD, disrupting the binding site for HveA/HVEM but not the binding site for HveC/nectin/Prr-1. The recombinant and wild-type viruses replicated in a variety of cell lines that expressed HveC/nectin/Prr-1. The recombinant failed to replicate in a cell line that does not express HveC/nectin/Prr-1, but did replicate in a derived clone that expressed the IL-13 receptor. Although mutants that are unable to enter cells through HveA/HVEM, but which have preserved HveC/nectin/Prr-1-mediated entry functions, have been previously described, this study provides preliminary evidence that gD engineering might allow viral entry through generation of a novel binding specificity. However, the crucial test of this strategy for viral targeting will be whether viral entry functions of gD remain preserved after disruption of the HveC/nectin/Prr-1-binding site, an observation that was notably absent from this report. Ongoing studies will determine whether engineering a novel binding ligand into the gD N-terminus will substitute for gD binding to HveA and HveC and preserve the role of gD in penetration.

An alternative strategy might use a bispecific soluble adapter molecule to bind to the HveC/nectin/Prr-1 site of gD, inducing the viral entry cascade. The molecule would also contain a binding site for a specific cellular ligand, thus providing the following 3 properties essential for targeted entry: (1) targeted cellular binding, (2) promotion of viral fusion, and (3) blocking the gD HveC/nectin/Prr-1-binding domain that would enable entry to nontargeted cells expressing this receptor. We have shown proof of principle that viral entry might be triggered by a soluble HveC/nectin/Prr-1 fragment containing the gD-binding domain (Bai and Glorioso, unpublished data, 2002) in the absence of a specific interaction between the soluble adapter and the cell surface.

Binding of gD to its cognate cellular receptor is followed by gB/gH/gL-mediated virion envelope–plasmalemmal fusion. As might be predicted, mutants deleted for gH/gL or gB are defective for virus penetration but are not defective in attachment (1,2,109). Furthermore, antibodies directed against gB, gH, or gL are able to prevent entry without interfering with attachment. The mechanisms of viral envelope–plasmalemmal fusion remain uncertain. In the absence of a specific role ascribed to a cellular receptor, it seems unlikely at present whether this part of the cell entry cascade will be amenable to manipulation resulting in targeted cell entry.

IV. VECTOR TRANSGENE EXPRESSION

A. Lytic Gene Promoters

We have explored the activity of many viral and cellular promoters in the background of replication-defective viral vectors (47,110–118). These lytic gene promoters display transient activity in both neuronal and nonneuronal cells. Thus, viral IE promoters are effective for applications that require only transient transgene expression. The human cytomegalovirus (HCMV) IE gene promoter produced vigorous transgene expression for up to 21 days post infection in vivo (Ramakrishnan and Fink, unpublished data, 1991) and in neuronal cell cultures in the background of a vector deleted for ICP4, ICP27, and ICP22 (41). Studies in rabbits and primates have demonstrated transgene expression under control of the HCMV promoter for at least 1 year following infection of rabbit joints (119). Other promoters, such as SV40 and various retroviral LTRs, are also transiently active (113,120) following infection of brain. Cellular promoters such as the muscle-specific muscle creatinine kinase (MCK) enhancer support muscle-specific expression in myotubes in culture (Akkaraju and Glorioso, 1991). Several reports have described long-term transgene expression from similar promoters in neurons (121–124), but expression declined significantly after the first week and may represent activity of aborted reactivation events. However, many therapeutic applications will require prolonged transgene expression from latent genomes. For this purpose, we and others have studied the native latency gene promoters in considerable detail.

B. Biology of Latency and the LAP

Following infection of epithelial cells of skin or mucous membrane, viral particles come into contact with sensory neuron axon terminals in which the particle is transported along mi-

crotubules to the nerve cell body where the viral DNA enters the nucleus (125,126) (Fig. 2C). Although the virus can express lytic functions in sensory neurons (127–129), lytic gene expression is curtailed through a set of largely undefined molecular events and the virus enters a latent state. Latency is typified by expression of a series of latency-associated transcripts (LATs) from the repeat regions flanking the long unique segment (U_L) of the viral genome partially antisense to and overlapping the 3′ end of ICP0 mRNA (130–132) (Fig. 2D). Two colinear, nonpolyadenylated (poly A^-) LAT RNA species of 2.0 and 1.5 kb (133,134), which accumulate in the nuclei of latently infected neurons (135), are stable nonlinear intron lariats derived from a large, unstable 8.3-kb polyadenylated primary transcript (136–140). The virus can remain latent for the life of the individual, although sporadically viral genomes may reactivate in response to a variety of stimuli including immune suppression, ultraviolet light, fever, and stress (126,141).

The promoter/regulatory region that controls LAT expression is of interest to gene therapy applications because that promoter remains active during latency when all other viral promoters are silenced. LAT expression is differentially directed by 2 LAPs, LAP1 (142–145) and LAP2 (145–147). LAP1, predominantly responsible for LAT expression during latency (142,145), is located 5′ proximal to the unstable 8.3-kb LAT. LAP2 is primarily responsible for LAT expression during lytic infection, but is also capable of driving low-level expression of LAT in the absence of LAP1 during latency (145) and is located immediately upstream to the 2.0-kb LAT intron (145–147). Deletion of both LAP1 and LAP2 completely eliminates expression of detectable levels of LAT during latency in animals (145), demonstrating that both promoters contribute to LAT expression during latency in neurons in vivo. The continuous expression of the LAT region of the HSV genome during neuronal latency suggests that it should be possible to exploit the LAT promoter to express therapeutic genes from latent viral genomes in neurons. Both LAP1 and LAP2 have been employed to achieve long-term transgene expression from the HSV vector genome during latency. For example, a LAP1-β-globin recombinant produced transgene expression in murine peripheral neurons during latency (142), but the level of product decreased over time (148). Other examples include recombinants with LAP1 driving expression of β-glucuronidase (149), NGF (148), β-galactosidase (123,146,148,150), or murine α-interferon (113) that either displayed a similar expression pattern or were not active in latently infected animals (113,123,148). These data suggest that LAP1 may lack the *cis*-elements required for long-term transgene expression in the context of the HSV viral genome. However, long-term transgene expression was achieved when LAP1 was juxtaposed to the Moloney murine leukemia virus (MoMLV) promoter (123), unlike recombinants employing either LAP1 or the LTR alone, suggesting that the elements responsible for extended expression lie elsewhere within the LAT promoter/regulatory region. These *cis*-acting elements may be complemented by elements within the MoMLV promoter, thus allowing transgene expression to continue during latency. When LAP2 was added to the LAP1-reporter cassette in the ectopic site within the genome, long-term expression was restored although the transcription start site was not determined (150). The LAP1–LAP2 complex was capable of driving long-term transgene expression when a lacZ reporter gene cassette was introduced into the LAT intron in the native LAT locus [(151), Chen and Glorioso, unpublished data, 1995] or when a LAP2-lacZ expression cassette was present in an ectopic locus within the viral genome (147). We have also shown that a LAP2-NGF cassette present either in the tk or Us3 loci of the vector expressed this gene product in latently infected rodent neurons both in culture and in vivo (111). Although, expression of β-galactosidase from the LAP-lacZ vectors could be detected in neurons of the mouse peripheral nervous system (PNS) for up to 300 days [(147), Chen and Glorioso, unpublished data, 1995], prolonged expression in the central nervous system (CNS) was at very low levels detectable only by reverse transcriptase-polymerase chain reaction (RT-PCR) techniques (Chen and Glorioso, 1995). Recently, we have determined that both the HCMV IE gene promoter and LAP2 are capable of long-term NGF expression in rabbits following intra-articular injection with a replication-defective HSV vector (119). These unanticipated findings will be important for designing treatment regimens where continuous therapeutic protein production will be required to achieve a therapeutic outcome.

C. Drug-regulated Promoters

For many applications, it may be desirable to regulate expression of a therapeutic transgene in vivo, either to limit toxic effects from high-level expression or more closely resemble physiological expression profiles. We created a viral vector with regulatable transgene expression by using an autoregulatory loop that consisted of a promoter with 5 tandem copies of the 17-bp Gal4 DNA recognition element that could be transactivated by vector-encoded chimeric Gal4/VP16 protein (Fig. 7A), based on the ability of the chimeric protein to transactivate promoters containing this site (152–154) despite the repressive presence of nucleosomes (155,156). The constitutive Gal4/VP16 transactivator was able to induce transgene expression from a Gal4-sensitive minimal promoter in the background of the virus (115). Regulation was achieved by replacing the constitutive transactivator with a chimeric molecule consisting of the hormone-binding domain of the mutated progesterone receptor fused to the transactivation domain of VP16 and DNA-binding domain of Gal4 (157,158). In the presence of the progesterone analog RU486, the inactive chimeric transactivator assumes a conformation allowing it to bind to and transactivate the Gal4 recognition site-containing promoter driving transgene expression. Compared with control (Fig. 7B), the completion of the autoregulatory loop following administration of RU486 (Fig. 7C) resulted in substantial enhancement of expression of the transgene in the CNS (115) (Fig. 7D). Following infection of the rat hippocampus with the regulatable virus, levels of viral vector-derived transgene expression are stimulated by administration of the inducing agent RU486 (159).

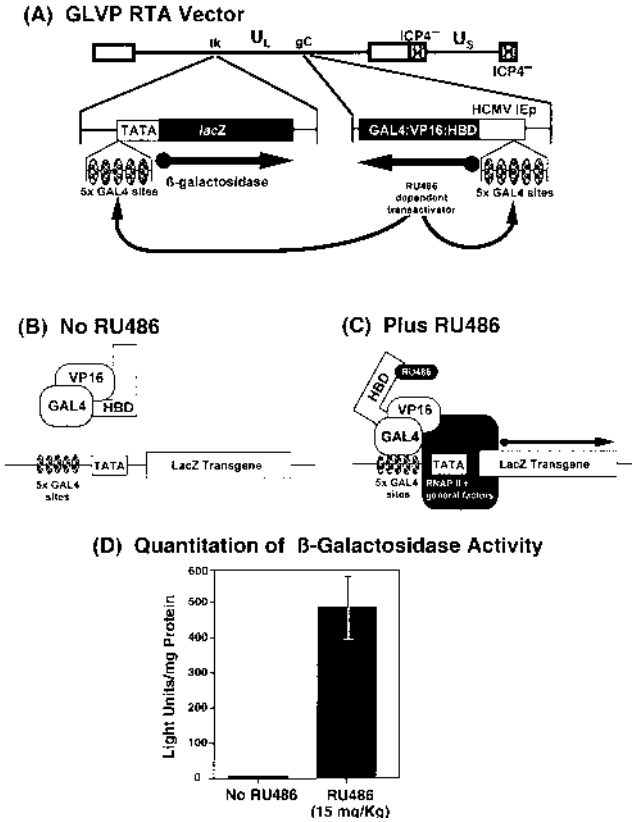

Figure 7 In vivo regulated transgene activation from HSV vectors by drug-inducible recombinant transactivator (RTA). (A) The GLVP RTA vector contains the inducible chimeric transactivator GAL4:VP16:HBD, composed of the yeast GAL4 DNA-binding domain fused to the HSV VP16 transactivation domain fused in frame to a mutant form of the progesterone receptor hormone-binding domain, which can be activated by RU486. In addition, the vector contains a promoter-reporter cassette ($GAL4_5TATA$-lacZ) that is responsive to activation by the transactivator (RTA). (B) In the absence of the drug, the GAL4:VP16:HBD recombinant transactivator cannot bind to the GAL4 sites in the minimal TATA box promoter, resulting in no expression of the β-galactosidase transgene. (C) Following administration of RU486, the tripartite transactivator undergoes a conformational change that allows the RTA to bind to the GAL4 binding sites to yield β-galactosidase expression. (D) Quantitation of β-galactosidase transgene expression in the presence of RU486 displays activation of the transgene promoter by the inducible transactivator following injection of the vector into rat CNS.

V. APPLICATIONS

A. HSV Gene Transfer for Neuropathy and Pain

In some sense, the most natural target for the therapeutic use of HSV-mediated gene transfer is the PNS. Recombinant replication-defective vectors, such as wild-type HSV, target with high efficiency to dorsal root ganglion (DRG) neurons following subcutaneous inoculation, a process that benefits from high-level expression of the HveC HSV receptor on sensory nerve terminals in the skin (160) and specific interactions between capsid and tegument proteins and dynein to mediate retrograde axonal transport along microtubules (161), followed by injection of the DNA through a nuclear pore into the nucleus. We have used HSV-mediated gene transfer to the PNS in rodent models of peripheral neuropathy and in the treatment of pain. The underlying rationale for using HSV-mediated gene transfer in these 2 conditions is similar. In both cases, peptides of proven efficacy have been identified. Peripheral neuropathy can be prevented in animal models by treatment with neurotrophic factors, and pain can be substantially ameliorated by the delivery of opioid peptides. However, it is difficult to deliver these short-lived peptide factors in adequate doses to achieve therapeutic effects without causing intolerable side effects that result from the widespread expression of the cognate receptors throughout the nervous system and in nonnervous structures in the body. Targeted gene delivery using HSV vectors can be used to overcome these limitations.

1. Neuropathy

We used subcutaneous inoculation of HSV-based vectors in the foot to deliver and express neurotrophic factors in DRG neurons in order to protect against peripheral nerve degeneration in models of neuropathy. We constructed vectors to express neurotrophin 3 (NT3) under the control of a fusion LAP2 HCMV promoter (vector QL2HNT3) and NGF under the control of the HCMV or LAP2 promoters (vectors SHN and SLN) (111). In the pyridoxine (PDX)-intoxication model of large-fiber sensory neuropathy, rats transduced by subcutaneous inoculation into the foot with QL2HNT3 and then intoxicated with pyridoxine PDX demonstrated a dramatic preservation of sensory nerve function, measured by sensory nerve recording (foot sensory amplitude and velocity), h-wave reflex, and behavioral assessment of proprioceptive function [Figs. 8A, B, and C from (162)]. Morphometric examination of the sciatic nerve demonstrated a substantial and statistically significant loss of predominantly large myelinated fibers in control vector-treated PDX-intoxicated animals, which was prevented by the NT-3 expressing vector QL2HNT3 (162). The NGF-expressing vector SHN provided a similar protective effect in the same model.

Mice rendered diabetic by the injection of streptozotocin develop a pure sensory neuropathy, manifest by reduction in the evoked sensory amplitude. Injection of either vector SHN or vector SLN 2 weeks after the onset of diabetes protected the animals from the development of neuropathy, measured by the foot sensory amplitude at 6 weeks of diabetes (163). This protective effect persists through 6 months of diabetes (Goss and Fink, unpublished data, 2002). In addition, expression of neuropeptide (calcitonin gene-related peptide and substance P) genes in the in the DRG was preserved in the vector-inoculated animals, demonstrating protection of the neuro-

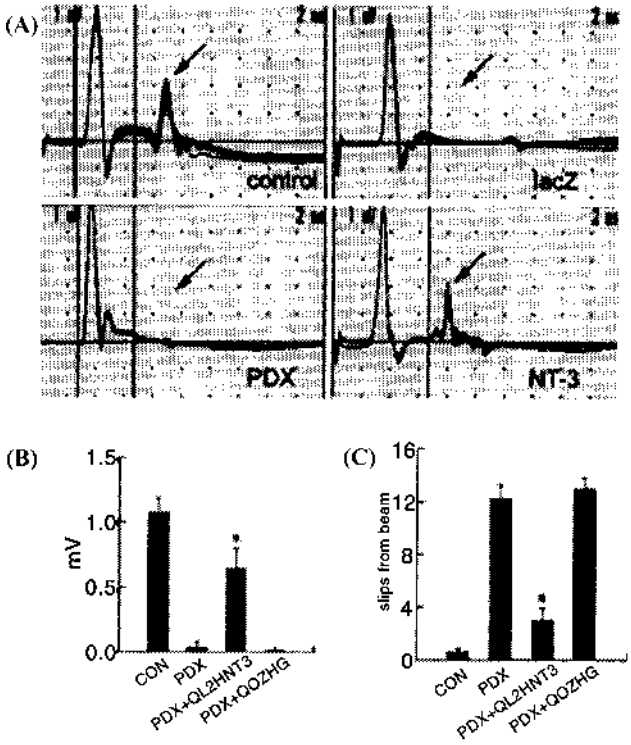

Figure 8 Neuroprotective effects of HSV vector-mediated NT-3 expression. (A) Examples of individual M wave and H reflex (arrows) recorded from the gastrocnemius muscle after stimulation at the sciatic notch. Control is naive animal, PDX is an animal treated with pyroxidine, lacZ is treated with PDX and the control lacZ vector QOZHG, and NT-3 is treated with PDX and the NT-3 expression vector. (B) The mean H-wave reflex amplitude from the measurements in A. (C) Behaviorial analysis of propriceptive function. Rats were trained and tested on a 3-cm beam with the number of slips from the beam measured during a 3-minute session. The PDX-treated animals have great difficulty traversing the beam; however, treatment with the NT-3 expression vector prevents this deficit. (From Ref. 162.)

chemical phenotype (163). We have observed similar protective effects against the development of neuropathy using the same vectors (QL2HNT3, SHN, and SLN) in the rodent model of neuropathy resulting from administration of the chemotherapeutic drug cisplatin (164).

In each model tested, the HSV vector coding for a neurotrophic factor has been inoculated prior to the onset of neuropathy. We have not examined whether the vectors might be used to enhance recovery from neuropathy, but achieving prevention alone would be a major advance in the treatment of neuropathy. Most sensory neuropathies develop gradually over a period of months to years; patients with diabetes, for example, characteristically present with loss of sensation in their toes, but there is no available therapy that will prevent progression of neuropathy. Gene transfer at that stage, if it prevented the development of a severe sensory neuropathy, would represent a substantial advance (165). Neuropathy caused by chemotherapeutic drugs represents one model in which the onset of neuropathy is temporally defined and pretreatment is possible. This type of neuropathy may serve as a convenient model for the first human trials of such therapy.

2. Pain

The treatment of pain uses the same approach, subcutaneous inoculation to transduce DRG neurons, to achieve local expression of peptides in sensory neurons. Wilson first constructed a replication-competent HSV-based vector to express proenkephalin (117). Subcutaneous injection of that vector into the dorsum of the foot in mice produced an antihyperalgesic effect that could be demonstrated by an increased latency to withdraw the foot from noxious heat after sensitization of C fibers by application of capsaicin, or sensitization of Aδ fibers by application of dimethyl sulfoxide (117). Although no antinociceptive effect was demonstrated in the absence of prior sensitization using the experimental paradigms reported, substantial expression of methionine-enkephalin could be demonstrated by immunocytochemistry (117). A replication-competent HSV-based vector expressing proenkephalin reduced pain in a rodent model of arthritis (166).

We extended these results by constructing a replication-incompetent HSV vector with the human PE gene (coding for 6 copies of met-enkephalin and 1 copy of leu-enkephalin) in the tk locus of the ICP4$^-$ recombinant d120 (designated vector SHPE). Inoculation of the vector into the foot transduced DRG neurons, and expression of the human proenkephalin gene and RNA was confirmed in DRG by PCR and RT-PCR. Expression of the peptide could be demonstrated in DRG neurons transduced in vitro by immunocytochemistry (167). We examined the biological activity of enkephalin (ENK) expression of SHPE transduction in the formalin model of inflammatory pain. Rats injected with SHPE 1 week prior to formalin testing showed a significant reduction in nocisponsive behaviors during the delayed phase (30–60 min after formalin injection). The antinociceptive effect was reversed by intraperitoneal naloxone (50 mg/kg i.p.) and by intrathecal administration of naltrexone. The effect of SHPE transduction waned over time. Animals tested 2 weeks after SHPE transduction showed a smaller reduction in nocisponsive behavior than those tested 1 week after transduction, and by 4 weeks after transduction there was no longer any significant antinocisponsive effect. Reinoculation of SHPE at 4 weeks reestablished the antinociceptive effect, measured 1 week later (5 weeks after the initial inoculation) by formalin test. These results suggest that the loss of effect was not a result of tolerance and demonstrate that prior inoculation with the nonreplicating vector does not prevent the reapplication of therapeutic gene transfer with the HSV vector.

To determine whether the effect of vector-mediated ENK release in DH is restricted to the distribution of transduced neurons projecting to DH, we repeated the experiment, but compared the pain response in animals injected with formalin

ipsilateral to vector footpad inoculation or contralateral to vector inoculation. There was a significant reduction in nocifensive behavior in the delayed phase of the formalin test when formalin was injected ipsilateral to SHPE inoculation 1 week after vector inoculation. There was no reduction in nocifensive behavior observed in animals injected with formalin into foot contralateral to the SHPE inoculation. SHZ injection had no effect on nocifensive behavior observed in either foot. In conjunction with the evidence that intrathecal naltrexone blocks the vector-mediated effect, these results suggest that vector-mediated ENK released from the central terminal of transduced primary afferent neurons to act locally in DH in the distribution of the central projection of those neurons.

We next examined the antinociceptive effect of the PE-expressing vector in the spinal nerve ligation (SNL) model of neuropathic pain, established according to the method described by Kim and Chung (168). Under chloral hydrate anesthesia, the left transverse process of L6 was removed, and the L5 spinal nerve was gently isolated and ligated tightly with 6.0 black silk suture distal to DRG but proximal to the formation of the sciatic nerve. Tactile threshold was determined using calibrated von Frey filaments (169,170). Subcutaneous inoculation of SHPE 1 week after SNL caused a significant reduction in tactile allodynia in the injected foot (171). The antiallodynic effect was maximal 2.5 weeks after vector inoculation and declined over the subsequent 4 weeks. Reinoculation of the vector reestablished the analgesic effect (171), which then persisted for approximately 6 weeks. The antinociceptive effect of SHPE was reversed by intraperitoneal naloxone. These results are consistent with published evidence, suggesting that mechanical allodynia in SNL models of neuropathic pain results from aberrant electrical activity in undamaged fibers from DRG, that project along with degenerating fibers from the ligated sensory root into the same peripheral nerve (172). The time course of the biological effect of transgene expression from this vector, in which the HCMV IEp is used to drive transgene expression suggests that at least 6 weeks of expression may be achieved in transduced DRG neurons.

Vector transduction enhanced the effect of morphine. Uninoculated animals with neuropathic pain responded to intraperitoneal morphine with an ED_{50} of 1.8 mg/kg (CI_{95} 1.1–2.8 mg/kg). Animals inoculated with SHPE 1 week after SNL and tested with morphine 1–2 weeks later demonstrated a reduction in the ED_{50} of morphine to 0.15 mg/kg (CI_{95} 0.06–0.34 mg/kg, $P < 0.05$) (171). Inoculation with SHZ had no effect on the response to morphine. In addition, twice daily administration of morphine resulted in a graduate decrease in the antiallodynic effect in the neuropathic rats, with no detectable effect by day 7. Animals that had been inoculated with SHPE 1 week after SNL showed a significantly greater response to morphine at days 1 and 2, consistent with the interaction of SHPE with morphine, and continued to exhibit an antiallodynic effect at day 7, when morphine alone was no longer effective (171).

Pain resulting from cancer metastatic to bone has features of both inflammatory and neuropathic pain. To evaluate the potential therapeutic effect of HSV-mediated gene transfer and expression of PE in pain due to cancer, we tested the vector in the osteogenic sarcoma model in the mouse (173). Tumor-injected mice demonstrated spontaneous pain, increasing to 2 weeks after tumor inoculation and remaining at the same level up to 4 weeks postinoculation. Subcutaneous inoculation of SHPE 1 week after tumor implantation resulted in a substantial and significant reduction in spontaneous behavior recorded 2 and 3 weeks after tumor implantation (1 and 2 weeks after vector inoculation) (174). The analgesic effect of the vector was reversed by intrathecal naltrexone.

Taken together, the results of studies demonstrate that a nonreplicating genomic HSV vector expressing PE is antinociceptive in models of neuropathic pain, inflammatory pain, and pain resulting from cancer in rodent models. A proposal for the first human trial employing these vectors in the treatment of intractable pain resulting from cancer metastatic to a vertebral body was presented to the Recombinant DNA Advisory Committee at the National Institutes of Health in June 2002. In the near future, we should be able to determine if this approach will be as successful in treating the human disease as it has been in the animal models.

B. Cancer

Cancer gene therapy may offer a treatment modality to patients who have exhausted all other standard treatment regimens such as surgery, chemotherapy, and radiation therapy. There are a number of considerations in applying gene therapy to the treatment of cancer that include the selection of the appropriate therapeutic gene(s), the specific effect or mechanism, target tissue, and method of gene delivery. The overriding problem is that cancer is generally a systemic disease, and thus, even if gene transfer is effective in destroying a tumor locally, metastases will promote continued disease.

Strategies to treat cancer by gene therapy can be considered in 3 categories: (1) tumor cell destruction using conditionally replicating viruses that selectively replicate in and kill tumor cells (175,176) compared with the surrounding normal tissue, (2) tumor cell destruction by expression of transgenes whose products induce cell death, or sensitize the cells to chemo- (177) or radiation therapy (178), and (3) tumor vaccination through expression of transgenes whose products recruit, activate, or costimulate immunity or provide tumor antigens. The latter approach is more likely to be effective in treating metastatic disease. Because these strategies are complementary, it has also been suggested that they can be used in combination. Examples of these various approaches include the use of (1) prodrug-activating genes such as thymidine kinase (TK) or cytosine deaminase (179); (2) cytokines such as TNF-α, γIFN and various interleukins (180); (3) MHC products such as costimulatory molecules (B7.1) (181–183); (4) allotypic class I or class II molecules (181–184); and (5) tumor antigens (185,186), which together may assist in the recruitment and activation of nonspecific inflammatory responses (187) or the induction of tumor-specific immunity.

The first strategy involves the use of vectors that are replication competent, but depend on attributes unique to the tumor cell to support viral growth. For example, E1b-deficient adenoviral vectors can replicate in tumor cells mutant for p53 but generally not in normal cells (175,188–190). Thus, the intent of this strategy is to provide a mechanism for virus spread locally in the tumor to increase the number of infected tumor cells. However, this treatment is limited to p53-defective tumors. Similarly, HSV vectors have been engineered that replicate in dividing cells, such as tumor cells, but not in normal neurons. The use of conditional replication-competent viruses could in theory allow for spread in tumor tissue without damaging normal brain, thereby increasing the specificity and effectiveness compared with nonreplicating vectors that express transgenes that augment tumor cell killing. HSV vectors of this type include mutants lacking the 34.5 gene, which is required for growth specifically in neurons (191–193). Deletion of this gene alone (1716), or in combination with the UL39 ribonucleotide reductase (RR) large subunit (G207), creates viruses that are highly compromised for their ability to replicate in and kill neuronal cells, yet that retain the ability to replicate in and kill dividing tumor cells.

Although these vectors were originally used to treat animal models of malignant glioma (194–198), they have now been employed to treat breast (199,200), lung (201,202), head and neck (203), melanoma (204,205), colorectal (206–209), prostate (210–213), ovarian (214,215), peritoneal (216–218), bladder (211,219), renal (220), cervical (221), and gallbladder (222) tumors in various animal models, demonstrating their utility. Moreover, in addition to their application for direct tumor cell killing, they have also been employed in tumor vaccination models (201,206,209,223,224). They have also been employed to augment the host immune response to the tumor by expressing either cytokines such as IL-12 (225) or immunomodulatory molecules such as B7–1 (226). In addition, they have also been used in conjunction with suicide gene therapy (227), low-dose ionizing radiotherapy (208,221), and chemotherapeutic agents like cisplatinum (202,228).

The safety and biodistribution of these vectors following intracranial administration has been examined in both rodents and nonhuman primates. The G207 vector deleted for 34.5 and UL39 (RR) displayed a very good safety profile (229–232) in both mice and nonhuman primates (rhesus macaques and Aotus nancymae), and vector remained locally surrounding the injection site. In addition, injection of vector was unable to reactivate endogenous resident wild-type HSV (230,233), a major issue concerning the use of these vectors in a human population that may already possess latent virus, again demonstrating the safety of HSV as a therapeutic vector. Safety studies using the 1716 vector have shown that deletion of 34.5 alone does not necessarily render the vector nontoxic. Vector injection into the ventricles of immune-deficient nude mice resulted in animal fatalities (234), or persistent ependymal cell loss in immune-competent mice (235), with virus spread to distal sites being observed in immune- competent rats (236). Another major question that needs to be addressed with the use of HSV vectors in a human population that is 60% to 90% seropositive for the virus, concerns whether preexisting immunity to the virus will prevent effective therapeutic treatment following vector administration. Although prior immunized animals displayed both humoral and cell-mediated responses to the virus, these responses did not significantly alter the ability of the vector to kill tumor cells and increase animal survival in preimmune vs. naive animals (218,228,237).

Based on these preclinical studies, two phase I clinical trials have now been reported using conditionally replicating HSV vectors. These studies have demonstrated the feasibility of inoculating recombinant HSV viruses into glioblastomas. No evidence of viral encephalitis or reactivation of wild-type virus was seen in either study. Furthermore, no adverse events could be unequivocally attributed to the vectors. One study, which used the G207 virus deleted for the 34.5 neurovirulence gene and the UL39 ribonucleotide reductase gene, showed safety up to a dose of 3×10^9 pfu (176). A second study using a 34.5 mutant with a complementing mutation in the US11 gene, which restores the growth characteristics of wild-type virus without reversing the attenuated neurovirulence phenotype of the 34.5 mutant, showed safety to a final dose of 10^5 pfu (238). A total of 30 patients were treated in the 2 trials. These trials were not designed to test whether the vectors were efficacious in the treatment of glioblastoma (GBM); phase II efficacy studies are ongoing.

The second approach to treat cancer involves suicide gene therapy (SGT) for the treatment of cancer in experimental animals and in phase I human clinical protocols (177,239–243). This strategy uses the bystander destruction of tumor cells mediated by a variety of mechanisms other than virus spread, including the recruitment of natural killer (NK) cells by expressing the appropriate cytokines, the activation of anticancer drugs at the tumor site that kill multiple tumor cells in addition to those transduced by the vector, and the use of antigens and cytokine-expressing genes to elicit specific antitumor immunity. Transfer of the HSV gene TK into tumor cells results in tumor cell death when combined with the antiviral drug ganciclovir (GCV). TK has been shown to convert the prodrug into a toxic nucleoside analog that, upon incorporation into nascent DNA, results in the interruption of DNA replication by chain termination. A uniquely powerful characteristic of the TK-GCV approach is that only a small fraction of the tumor cells need to be transduced with the suicide gene to result in significant antitumor activity, an activity known as the ''bystander effect'' (239,240,243–245). It has been demonstrated that cell-to-cell transfer of activated GCV via gap junctions between transduced tumor cells and untransduced neighboring cells is a major mechanism of the bystander effect (246–249). A variation on this strategy is to introduce cell lines into the tumor site that produce TK-expressing retroviruses that then infect tumor cells locally. Although setting up virus production factories in this manner is logical, the practice of this strategy has been disappointing because the xenogeneic producer cell lines induce rapid inflammatory processes that lead to brain swelling and little detectable gene transfer (250). Although these TK-GCV SGT strategies suffer from limitations, approaches of this nature are under evalua-

tion for efficacy in phase I–II clinical trials for patients with brain tumors; however, they have met with limited success.

In our initial experiments using replication-defective HSV vectors expressing HSV-TK for SGT, we employed both vectors deleted for single (ICP4$^-$) and multiple (ICP4$^-$/ICP27$^-$/ICP22$^-$) essential IE genes that expressed the TK gene from an HSV IE promoter to ensure its expression from the replication-defective virus backbone (Fig. 9A). We tested the ability of these TK overexpressing replication-defective HSV vectors to act as a treatment for established tumors in rodent glioma models and demonstrated significant increases in survival following administration of the HSV-TK vector and GCV (114,251). However, the magnitude of the bystander effect was inversely proportional to the overall toxicity of the vector. Thus, more cytotoxic vectors like the ICP4 deletion mutant resulted in the cytotoxic death of the transduced tumor cells before they were able to produce and release significant levels of modified GCV, thereby dampening the overall bystander-mediated killing (251). We have now seen similar results using vectors that express CD alone or in combination with HSV-TK (252), suggesting that further modifications will be required to achieve more effective tumor cell killing.

To augment the cell killing seen in SGT we have taken two approaches. In the first approach, we attempted to augment the bystander effect by altering the makeup of tumor–cell gap junction complexes. Connexins are the components of gap junctions (253) that play a major role in intercellular communication to control homeostasis and cell proliferation. Reduced intercellular communication through gap junctions has been regularly observed in transformed cells (254–256) and may be due to reduced connexin expression. Retrovirus-mediated introduction of the connexin 43 (Cx43) gene limited the growth of transformed cells that shared the characteristic of reduced gap junctional activity (257), and other studies have reported similar findings supporting the suggestion that connexins alone have tumor-suppressor activity (258–261). Gap junctions also play a critical role in the HSV-TK/GCV bystander effect by enabling the transfer of activated GCV from TK-positive to neighboring TK-negative cells (246,262–265). Tumor cells having reduced gap junction formation are less susceptible to the bystander effect (265), suggesting that transfer of connexin genes into these cells to restore or augment intercellular communication would improve the effectiveness of HSV-TK/GCV therapy. Indeed, it has been demonstrated both in vitro and in vivo that the bystander effect can be potentiated by the expression of connexin (264,266,267). The bystander effect requires connexin expression not only by the TK-positive GCV-activating cell, but also by the TK-negative recipient cell (265). This suggests that gene therapy approaches aimed at codelivery of connexin with HSV-TK to mediate an enhanced bystander effect may not be extremely effective against tumors that express no connexin at all. However, it may from a study of 17 cell lines measuring bystander effects and gap junctional activity (263); this will be an extremely small group. Furthermore, overexpression of connexin in the transduced cells will increase transfer of activated

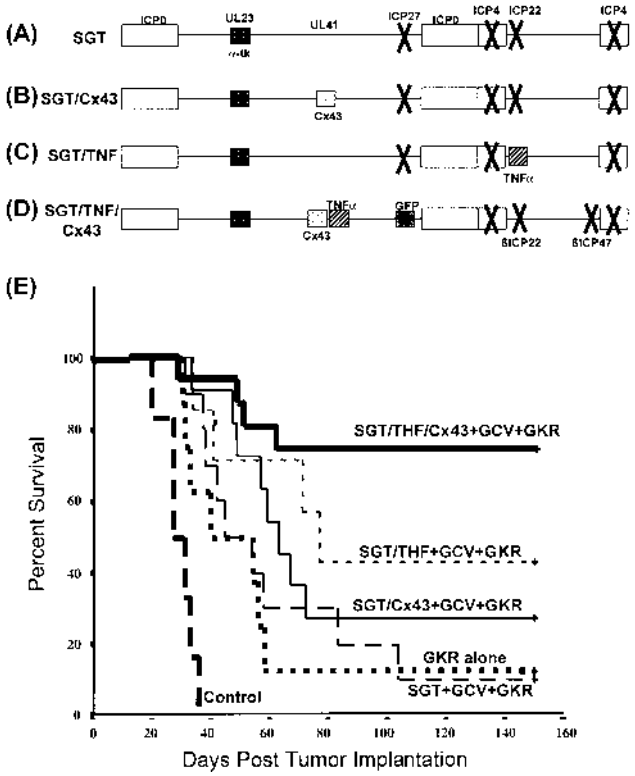

Figure 9 HSV-vector mediated tumor cell killing. Diagrams of replication-defective HSV-1 expression vectors for expressing (A) HSV-TK (SGT), (B) HSV-TK and connexin 43 (SGT/Cx43), (C) HSV-TK and TNF-α (SGT/TNF), and (D) HSV-TK, connexin 43, and TNF-α (SGT/TNF/Cx43). All vectors express ICP0 from both copies of the inverted repeat element flanking the unique long segment (UL) of the HSV genome, and TK as an IE gene from a copy of the ICP4 IE promoter replacing the native TK promoter in the UL23 gene locus. All vectors have the ICP4 and ICP27 genes deleted, and all but the SGT/TNF/Cx43 vector have inactivating deletions in ICP22. This vector has the IE genes ICP22 and ICP47 turned into early genes by mutation of the TAATGARAT sequences in these promoters converting these to early (β) genes. The SGT/Cx43 vector contains the Cx43 gene driven by the ICP0 IE promoter inserted into the UL41 locus. The SGT/TNF vector has TNF inserted into the ICP22 gene locus under control of the HCMV IE promoter. The SGT/TNF/Cx43 vector has both the ICP0p-Cx43 and HCMV IEp-TNF expression cassettes inserted into the UL41 locus. The ability of these vectors to destroy tumors and effect animal survival in the immunocompetent rat 9L tumor model was evaluated by intratumoral vector injection 3 days following implantation of the 9L tumor cells into the frontal lobe of Fisher rats. Animals received i.p. injections of GCV at the time of vector injections for 10 consecutive days and gamma-knife radiosurgery (GKR) at 2 days post vector injection.

GCV, providing even limited connexin expression in neighboring cells.

We tested the potential benefit of coexpressing connexin with HSV-TK for the treatment of glioblastoma (268). Human U-87MG tumor cells express detectable amounts of Cx43 and are sensitive to bystander killing in vitro, but this effect is not sufficient to control tumor formation with TK-transduced cells and GCV treatment alone (254). Replication-defective vectors ($ICP4^-/ICP27^-/ICP22^-$) that we engineered to express Cx43 (Fig. 9B) were able to enhance TK-GCV tumor cell killing in cultures of U-87MG tumor cells that express Cx43 (268). Thus, although U-87MG cells already showed a good bystander effect, the expression of additional Cx43 in a fraction of the population further enhanced the effectiveness of GCV treatment. We also were able to demonstrate a pronounced bystander effect with L929 cells (268) that did not show a bystander effect when infected with a TK-expressing vector, in agreement with recent findings by others (265). This indicated that vector-directed connexin expression enabled bystander killing among these otherwise bystander-resistant cells. Together, these results suggested that vector-directed Cx43 expression should be beneficial regardless of whether the target cells express significant levels of connexin.

These in vitro assays were extended to animals using an ex vivo flank tumor model and in vivo using animals bearing U-87MG tumors in the CNS (268). Dramatically, all animals implanted with Cx43 vector-infected cells and treated with GCV in were tumor free 1 week after cessation of GCV administration, whereas no other animal was tumor free at this time, including animals treated with Cx43 vector alone or the TK-expressing vector plus GCV. Moreover, all animals in this treatment group were alive at the end of the observation period (72d), whereas no animals in any of the other groups survived past day 41. Encouraged by these promising results, experiments were initiated to test the effectiveness of combined Cx43/tk gene delivery in vivo because the ex vivo approach enables one to infect every tumor cell that does not readily mimic the actual situation in human patients with glioblastoma. In the in vivo experiments, virus was injected directly into the tumor mass 3 days following tumor cell implantation and GCV was administered for 10 days after vector inoculation. In these studies, all animals in every treatment group died by day 50, whereas 50% of the Cx43-expression vector-treated animals survived past 50 days and one-third were still alive at the end of the study (70d), indicating a beneficial effect of connexin/tk gene codelivery in vivo.

In the second approach to augment SGT, we created vectors that express cytokines in the hope of stimulating a host response to the tumor. There has been a considerable amount of recent interest in using cytokine genes, costimulatory molecules, tumor antigens, and recruitment molecules to enhance the immune response to the tumor. Antitumor immunity should prove effective in treatment of metastatic cancer. The development of antitumor immunity could circumvent the need for replication-competent vectors because tumor-specific cytotoxic T lymphocytes constantly move through the brain parenchyma searching for target cells. A growing body of literature suggests that local expression of cytokines can enhance CTL activation at least in animal model systems and these bear testing in human brain cancer. HSV offers the potential for combinational gene therapy in this regard because multiple immunomodulatory genes can be recombined into the virus and comparatively tested (47).

Tumor necrosis factor-α (TNF-α) has been demonstrated to possess an array of antitumor activities, including potent cytotoxicity exerted directly on tumor cells (269), enhancement of the expression of HLA antigens (270) and ICAM-1 (271) on tumor cell surfaces, enhancement of interleukin-2 receptors on lymphocytes (272), and stimulation of such effector cells as NK cells, lymphokine-activated killer cells, and cytotoxic T lymphocytes (CTL) (272–276). However, despite this promising antitumor profile, the clinical use of TNF-α has been constrained by the toxicity of systemic TNF-α delivery (275,276). This problem could be minimized by local production of TNF-α at the site of tumor growth, which may allow for effective use of this cytokine as an antitumor agent. Furthermore, TNF-α has a radiosensitizing ability that could optimize its antitumor effects. In an effort to augment the effectiveness of HSV-TK-mediated suicide gene therapy, we created the replication-defective, triple IE gene-deleted HSV vector ($ICP4^-$, $ICP27^-$, $ICP22^-$) that expresses HSV-TK and TNF-α (Fig. 9C).

In vitro studies demonstrated that high levels of TNF-α could be detected in the media of vector-infected cells during the first 24-h period, but this was followed by a precipitous decline in production on day 2 and subsequent days with the protein being no longer detectable on day 7 (114). The bioactivity of TNF-α produced in this experiment was tested by exposure of cultured TNF-α-sensitive L929 fibrosarcoma cells to medium collected after the first day of infection. A dramatic reduction in cell viability was observed for cells treated with medium from TNF vector-infected cells, and this reduction was essentially identical over time to that seen with unconditioned medium supplemented with 10 ng recombinant TNF-α protein (114). These results demonstrated that TNF-α produced by the TNF vector was biologically active and comparable in specific activity to recombinant TNF-α. We then determined that intracellular production of TNF-α could enhance HSV-TK/GCV-mediated cell killing of both the TNF-α-sensitive L929 cell line, as well as the TNF-α-resistant U-87MG cell line. Although the mechanism is unclear, this enhancement indicated that the combination of vector-directed TNF-α expression and HSV-TK expression with GCV treatment could be beneficial not only against TNF-α-sensitive tumor cells such as L929, but also against TNF-α-resistant tumors.

To determine if the increased effectiveness of combination gene treatment evident in vitro could also be observed in vivo, we first tested the effect of intratumoral vector injection followed by GCV treatment on established L929 tumors (TNF-α sensitive) in the flanks of immune-competent mice (114). Tumor treatment with the TNF vector plus GCV resulted in significantly greater growth inhibition and extended animal survival compared with all other treatments. These results

demonstrated the promise of combination TNF/TK gene therapy for the treatment of TNF-sensitive tumors and added an incentive to test the same treatment against TNF-resistant tumors. However, the results in the U-87MG TNF-α-resistant intracerebral tumor model in immunodeficient mice were not as significant as those observed with the L929 model, although 2 of 14 animals in the TNF/GCV treatment group survived past 80 days (114). This reduced response in the U-87MG model may be due to the fact that the tumor cell killing mediated by TNF is only the result of the cytotoxic effects of TNF expression intracellularly and is not augmented by its ability immunomodulatory ability. Together, these results suggest that combined HSV-TK/TNF therapy is beneficial, but that a more effective strategy may be required.

Fractionated radiotherapy has been shown to confer a small but significant survival benefit to patients with glioblastoma. Unfortunately, the dose of radiotherapy that may be tolerated by the brain (about 60 Gy) is inadequate for tumor eradication. To circumvent inherent toxicity problems, techniques have been developed that allow focusing of radiation to the tumor bed, allowing a higher dose to be delivered (radiosurgery). This enables eradication of the central portion of the tumor, but does not allow delivery of an augmented radiation dose to the tumor periphery. Gamma-knife radiosurgery (GKR) allows for precise delivery of a single high dose of radiation to brain tumors without opening the skull. In this technique, tumors are targeted by the application of a tightly focused high-energy radiation field, which results in minimal collateral damage to the surrounding normal tissue. Radiosurgery has been used for boost irradiation of patients with malignant glial tumors, in addition to conventional wide-margin fractionated radiotherapy (277,278). Unfortunately, glioma cells are often seen invading the normal tissue surrounding the tumor, often migrating along normal white matter tracts (279,280). This feature of glioma is largely responsible for the inability to effect a surgical cure by resection and the correspondingly poor prognosis. We have therefore examined ways in which the response to radiotherapy may be enhanced by gene delivery. One such approach involves selectively sensitizing tumor cells to radiotherapy. This would confer a major advantage, in that the sensitized cells could be effectively killed by a low dose of radiotherapy that is not toxic to surrounding brain tissue. In this strategy, we combine GKR with the injection of replication-defective HSV vectors expressing HSV-TK, Cx43, and TNF.

In the first series of experiments, we used GKR in conjunction with vector-mediated TNF expression because TNF has previously been shown to have a synergistic effect with ionizing radiation when delivered as a recombinant protein (281–287), by plasmid-based delivery (288–292) or by using an adenoviral vector (293–297). Moreover, the TNF-α approach has proven safe and shown some efficacy in human phase I clinical trials (297,298). Experiments were carried out to determine whether HSV-TK/TNF gene transfer along with GCV treatment was more effective in the presence of low-dose gamma-knife radiation. Both the TNF/TK vector and gamma-knife radiation alone were effective in protecting a proportion of animals from tumor growth and animal death when used in the U-87MG model of glioblastoma in nude mice (299). The results using the TNF/TK vector with SGT and GKR demonstrated that the combination of TNF, GCV, and GKR was superior to other treatments, such that 89% of the animals in that treatment group surviving for the length of the study (75d) and 67% of the animals were found to be tumor free at 75 days (299).

We then extended these results to an immunocompetent tumor model that may more closely mimic the human disease by carrying out survival studies comparing the efficiency of HSV-TK, TNF, and Cx43 in combination with GCV, with and without radiosurgery using the 9L gliosarcoma model in immunocompetent Fisher 344 rats (Niranjan and Glorioso, unpublished data, 2002). In this study, GKR was found to enhance the survival of 9L intracranial tumor-bearing rats compared with the control (Fig. 9E); however, treatment resulted in an overall survival of 15%, similar to what has been reported in human patients (277,300,301). Combining the TNF and connexin-43 genes in a HSV-TK/ICP0 vector (Fig. 9D) further improved animal survival (Fig. 9E). Eleven of 15 animals treated with the HSV-TK/TNF/Cx43 vector, GCV, GKR survived for more than 150 days compared with 7 of 15 treated with TNF, GCV, and GKR. These results demonstrate that our most effective current strategy for the treatment of animal brain tumors involves the multigene vector, which simultaneously expresses TNF-α Cx43, HSV-TK, and HSV-ICP0, combined with radiosurgery and GCV treatment. This combination approach employs genes whose products that are tumorcidal (HSV-TK, TNF), augment this process by increasing the bystander effect (Cx43), sensitize tumor cells to radiation (TNF), and stimulate the host immune response to the tumor (TNF). This combination approach may also prove to be effective against metastatic disease, but remains to be tested in these animal models.

VI. SUMMARY AND FUTURE DIRECTIONS

This overview of HSV biology and gene transfer has focused on the use of highly defective HSV genomic vectors that are blocked very early in the virus lytic cycle. These vectors express few viral functions and are highly reduced in vector toxicity, even for primary neurons in culture that are readily killed by less-defective HSV vectors. Moreover, these vector backgrounds are suitable for expression of multiple transgenes or single large genes (e.g., dystrophin) in applications where expression of single- or multiple-gene products are required to achieve a therapeutic outcome (e.g., tumor cell killing, vaccination). Expression of these transgenes can be coordinated, even sequentially, using strategies similar to those employed by the virus to regulate its own genes. Expression can also be controlled by drug-sensitive transactivators, which may prove to be important for regulating the timing and duration of transgene expression. HSV vectors may be most suited for expression of genes in the nervous system where the virus has evolved to remain lifelong in a latent state. The highly

defective viruses deleted for multiple IE genes are able to efficiently establish resistance in neurons and serve as a platform for long-term gene expression using the latency-active LAP2 promoter system. These mutants can not reactivate from latency and cannot spread to other nerves or tissues following infection of cells. Delivery of these vectors requires direct inoculation of tissue to achieve direct contact with neurons. Ideally, HSV vectors would be most effective if infection could be targeted to specific cell types using enveloped particles defective for their normal receptor recognition ligands, but modified to contain novel attachment and entry functions. This area of research is still very early in development, and it remains to be determined to what extent this will be feasible. Finally, it should be emphasized that current viral delivery systems may each become reduced to highly defective transfer vectors, retaining only those elements required for vector DNA maintenance and transgene expression. Fortunately, the natural biology of many persistent viruses, including HSV-1, indicates that long-term vector maintenance will be possible and we continue to learn from the highly evolved biology of persistent and latent viruses in order to mimic their strategies for gene transfer and therapy.

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7

Lentiviral Vectors

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I. INTRODUCTION

Lentiviruses are a genus of the family *Retroviridae* (retroviruses) (1) that have been modified to be used as gene transfer agents. The major attraction of this class of vector is its perceived potency in transducing and permanently modifying nonreplicating cells, not only in tissue culture but also in animals and, it is hoped, eventually in humans. This chapter summarizes the opportunities and issues surrounding the use of lentiviruses as clinical vectors, and so tries to cover a lot of ground. The references provide examples and leads for further information, and are not meant to be comprehensive.

II. BRIEF HISTORY

Retroviruses are RNA viruses that reverse transcribe their genome into DNA and then integrate it in the host cell genome. The name was coined after the characterization of murine leukemia viruses (MLVs) and their life cycle in the late 1970s and early 1980s. The molecular understanding of the viral life cycle led to, among other things, the development of retroviral vectors based on these types of viruses (mainly C-type retroviruses such as MLV) (2) and their extensive use in the clinic (refer to http://www.wiley.co.uk/genetherapy/clinical/ and http://www4.od.nih.gov/oba/rac/PROTOCOL.pdf).

Human immunodeficiency virus 1 (HIV-1) was the first well-characterized lentivirus (3). The identification and characterization of HIV-1 overlapped with the development of retroviral vectors described above; however, extensive characterization of the HIV system occurred somewhat later than the development of the MLV-based systems. Because retroviral vectors were perceived to have safety issues of unknown proportions (4,5), it was not attractive at the time to add to the degree of difficulty by using a known human pathogen as the basis of such vectors. However, it was known (and rediscovered several times) that C-type retroviruses such as MLV do not efficiently transduce nonreplicating cells while lentiviruses can (6), and this property of MLV, although conferring potential specificity, made some desirable applications impossible or, at best, more difficult than anticipated. These desirable applications included ex vivo transduction of cells and in vivo applications, such as delivery to liver, brain, lung, bone marrow, and other tissues (the majority) where there was normally little or no cellular replication.

The first lentiviral vectors were constructed by Sodroski, Haseltine, and their collaborators (7,8) and were based on HIV-1, using natural envelope that conferred tropism for CD4+ T cells. In fact, most of these early vectors were replication competent and were produced in a manner similar to the ''wild-type'' virus. Soon after this, Page et al. (9) described efficient pseudotyping with nonlentiviral envelopes such as the amphotropic MLV envelope, but this was seen as a molecular tool for laboratory use. In the meantime, the use of pseudotyping with the vesicular stomatitis virus G (VSV-G) protein for C-type vectors (10) and, crucially, the ability to easily concentrate these pseudotyped vectors by centrifugation and resuspension (11) were described. This information, and further data from extensive characterization of the HIV system in the mid-1990s, led to the demonstration that VSV-G pseudotyped HIV vectors could be made, concentrated, and used to transduce rat neural tissues in vivo (12). Such transduction led to long-term expression. The ability to use these vectors efficiently and easily in a research lab setting sparked a good deal of activity and initiated the current intense activity in the field of lentiviral vectors.

III. TYPES OF VECTOR—MOLECULAR STRUCTURE AND FUNCTION

A. Different Lentiviruses as the Bases for Vectors

The genome of HIV-1 (3) is shown in Fig. 1, as are those of other lentiviruses [feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), simian immunodeficiency virus (SIV), bovine immunodeficiency virus (BIV), caprine arthritis encephalitis virus (CAEV)] that have been used to make vectors (13). The HIV viral genome encodes 9 proteins: the structural proteins, Gag, Gag-Pol, and Env, plus accessory proteins: Tat, Rev, Vif, Vpu, Vpr, and Nef (Table 1). The other viruses have some of the same accessory proteins and some others. All the viruses have a Rev/Rev responsive element (RRE) system that allows export of unspliced or partially spliced viral RNA from the nucleus. All the viruses appear to be able to infect nonreplicating cells. In general, the vectors and packaging systems have moved from the first generation (Helper genome with an envelope deletion) (12) through a second generation that eliminated some of the accessory proteins (14) to third-generation systems (e.g., 15–17), shown in Fig. 2 and realized for HIV and EIAV, where almost all the unnecessary accessory proteins have been removed leaving just Rev, or Rev and Tat only. Further refinements are described below. The most thorough understanding of structure and function is, of course, for HIV-1, and the most commonly used envelope is the VSV-G protein. Vectors from the different systems appear not to differ in potency a great deal, although it is hard to be definite about this because even those experiments that claim to do so may not compare the best of both systems (e.g., 18,19). In general, vectors generated by transient transfection on 293T cells with a VSV-G pseudotype give titers around 10^6 transducing units (TUs)/mL as measured by a β-gal assay in tissue culture on various cell types. If you can achieve this, then you can join the club!

B. Accessory Proteins

The functions of the accessory proteins have been intensively investigated for HIV-1, but remain incompletely understood (see Table 1 and Refs. 3,20,21). It is possible to delete or inactivate all of *vif*, *vpr*, *vpu*, and *nef* and still retain viral replication in tissue culture (21), and so it is believed that most of these play a role in human or animal infection and pathology. The information concerning accessory proteins in other lentiviruses is often derived by analogy from the HIV data. Nevertheless, as shown in Fig. 1, although SIV looks quite similar to HIV, the nonprimate lentiviruses (13,22) are not as similar and do not carry analogs to HIV *vpr*, *vpu*, and *nef*. The Rev, Tat, and Vif proteins and functions appear to be conserved, however, with the exception that EIAV has no Vif protein and FIV has no recognizable Tat, although some tatlike functions are performed by Orf A (23). In addition, the nonprimate lentiviruses carry other genes of unknown function (e.g., S2 in EIAV) and also encode a dUTPase in the Pol polypeptide. This level of complexity is fairly daunting, and the approach of vector makers has been somewhat cavalier, but also apparently successful so far. This approach has been to eliminate these functions from vectors and helper functions as far as possible and current vector systems usually incorporate only *rev*, and then only in the production stage. It is unknown whether expression of such accessory proteins could, under certain circumstances, influence the properties of the vectors in uptake, transduction, and expression in animals or clinical trial subjects. However, it is also true that some of these proteins are toxic, at least in tissue culture, and their functions are incompletely understood, so the expression of these functions adds an unknown level of risk for use in animals and clinical trial subjects. The bottom line is that the vectors appear to be quite potent without the accessory proteins.

C. Integration in Nondividing Cells

As noted in Section I, a key issue has been the ability of these viruses (and the derived vectors) to infect (or transduce) nondividing cells (3,6,12). This is achieved by the passage of the intact preintegration complex of nucleic acid and protein through the nuclear membrane by a mechanism that remains unclear. The preintegration complex appears to be less massive than for C-type viruses because most of the nuclear capsid proteins are removed in the cytoplasm, with the matrix (MA) peptide and reverse transcriptase (RT)/integrase (IN) peptides retained (24,25). Contribution to the nuclear entry function have been mapped to 4 different viral functions in HIV: the MA protein of the nuclear capsid (22,26,27); the IN molecule encoded as part of the reverse transcriptase polypeptide (22,28); the accessory protein, Vpr (22,29); and the central polypurine tract (22,30). It is clear, however, that not all these are needed for the integration to occur in nondividing cells (31–33) and, for example, in some vectors only the MA and IN contributions remain, but integration in nondividing cells proceeds efficiently. The central polypurine tract appears to make the vectors more efficient in some situations but not in all (33). The MA function by itself may not be sufficient to allow the nuclear transport to occur as attempts to transplant the function into MLV vectors allowed infection of replicating, but not of growth-arrested cells (32).

Nevertheless, the lentiviruses and lentiviral vectors do not transduce all cell types by any means, even when equipped with a pantropic envelope such as VSVg, and the efficiency can vary widely. For example, in the initial description of this property (12), it was clear that HIV vectors can transduce human macrophages more efficiently than MLV-based vectors. Nevertheless the efficiency with which this happens (as measured by the effective titer on those cells) is around 1% of the efficiency on replicating tissue culture cells such as HeLa cells (17). Another example for which the entire story is not yet clear is the transduction of liver cells in vivo following intravenous (IV) administration of the vectors to animal models (mainly mice). Some investigators have experienced difficulty transducing hepatocytes in vivo (34), but other pub-

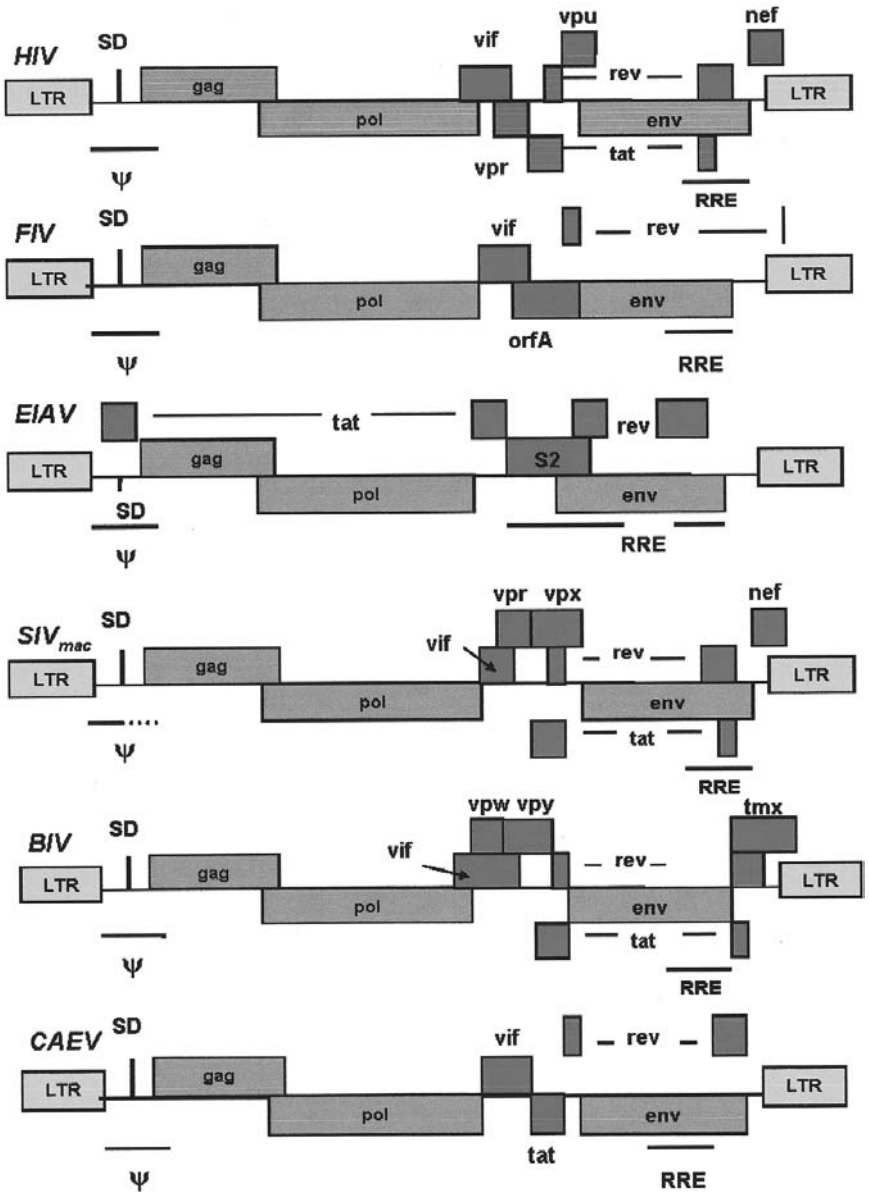

Figure 1 Genomes of lentiviruses that have been used as the bases for vector construction. The genomes are shown with the genes coding for the structural proteins in green, the accessory proteins in light blue, and the long terminal repeats (LTRs) in yellow. Ψ, packaging signal; SD, splice donor; RRE, rev responsive element. The diagram is not to scale. See the color insert for a color version of this figure.

lications do not report such a problem (35). This issue has been difficult to resolve because it is not clear how much exposure the total complement of hepatocytes in mature liver has to vector in the circulation, and therefore what ''efficiency'' to expect. In any case, once again, compared with MLV, the efficiencies of lentiviral vector-mediated transduction are much improved in vivo. This issue is discussed in more detail in Section V. There may be a block to hepatocyte transduction in vivo under some circumstances, but this is likely due to something other than the ability of the vector capsid to penetrate the nuclear membrane. It was originally suggested that the vectors may be better at transducing cells arrested in the cell cycle (G_2) rather than those that are in G_0/G_1 (12) or that have no possibility of replicating, but this

Table 1 Functions of HIV Accessory and Regulatory Proteins

Protein	Function
Tat	Activation of LTR transcription, other postentry functions, able to pass into neighboring cells intact, may set up neighboring cells for infection
Rev	Transport of unspliced or singly spliced message from nucleus to cytoplasm
Vif	Acts during virus assembly to make virus particle competent for subsequent infection—dependent on the cell type
Vpu	Facilitation of release of budding virus particles from surfaces of infected cells—cell-type dependent and not limited to HIV
Vpr	G2 cell-cycle arrest, nuclear transport, suppresses immune activation and apoptosis through regulation of nuclear factor kappa B, positive regulator of viral transcription and infectivity in primary human macrophages
Nef	Facilitates capsid disassembly upon infection, may down-regulate MHC

Source: Refs. 3, 20, and 21.

turned out not to be the case (36,37); in any case, the vectors are very efficient in transducing some postmitotic cells, such as neurons, in vivo (see below).

D. Packaging Signal

The packaging signal is the sequence that allows recognition and packaging of the genomic RNA into the viral capsid before the capsid proceeds to the internal surface of the coated pits where it picks up pieces of cellular membrane and some cellular surface proteins and buds from the cell (38,39). The packaging signal in general contains a set of ''lollipop'' RNA secondary structures that can be varied in sequence as long as the structure is maintained (22,40). This site is the area of the dimerization signal for inclusion of 2 viral genomes in the nascent particle, but this *trans*-interaction also appears to be structurally driven and not sequence specific. The packaging signal is recognized by nucleocapsid structural (NC) peptide from the Gag polyprotein to start formation of the viral capsid (41,42). It seems likely that this occurs through interaction with zinc fingers in the NC peptide.

The original definition of the packaging signal in HIV-1 was the 19 bp deletion between the LTR and the major splice donor by Lever et al. (8). Since then, the packaging signals for HIV-1 and -2, SIV_{mac}, EIAV, FIV, and BIV have been quite well defined (43–49). The packaging signal for these, as for most retroviruses, lies somewhere in the region between the 5′R region and includes the start of the *gag* sequence. It is bipartite, with the area around the splice donor probably being expendable although the spacing between the 2 segments is likely important. It is important to define this area

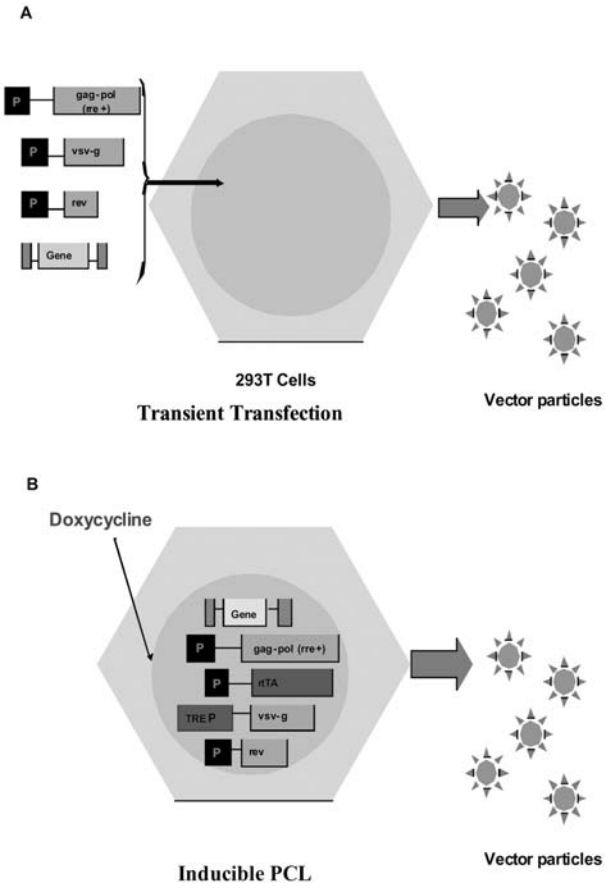

Figure 2 Processes for making vector preparations with third-generation packaging systems. (A) Transient process where the various elements shown are cotransfected into the recipient cell line that is usually 293T cells. This is a typical system that retains the need for Rev to get good expression of the Gag and Gag-Pol proteins, and to boost vector genome production. P, promoter, usually the CMV IE1 promoter. (B) Packaging producer cell line. All the elements necessary to make vector are in the cell line permanently. Doxycycline is used to induce expression of the VSV-G protein through the interaction with the constitutively expressed rtTA transactivator that induces expression from the TRE promoter after interaction with doxycycline or tetracycline. This is the ''tet on'' system designed by Gossen and co workers (117), but other inducible systems can be used. The blue hexagon represents the cell and the brown oval, the nucleus. The yellow box is the gene encoded by the vector, and the vector structure is simplified here with only the LTR regions shown in green. A more detailed diagram of a typical vector is shown in Fig. 3. See the color insert for a color version of this figure.

as closely as possible for 2 major reasons: (1) to obtain as high titers as possible, and (2) to identify and minimize obligate areas of homology between the vectors and the packaging constructs because these have been shown to lead to homologous recombination between these sites.

Homologous recombination reunites elements that were separated during the design of the vector system and could be a first step on the road to generating a replication-competent entity. In general it is easier to define what is necessary (by deletion analysis) than what is sufficient. The latter requires a more complicated molecular manipulation and the results are usually not black and white. From the viruses' point of view, the inclusion of *gag* sequence in the packaging signal makes sense because it allows for selection of full-length unspliced genomic RNA for encapsidation. From the vector makers' point of view, this is a nuisance because the *gag* sequence provides difficult-to-avoid sequence homology to the *gag* sequence in packaging plasmids provided in *trans* during vector production. Parenthetically, HIV-2 (and hence possibly SIV_{mac} because these 2 viruses are closely related) is reported to be an exception and the gag sequence may be a relatively unimportant part of the packaging signal (44). The *gag* recombination issue for vector construction could be addressed by simply leaving out part of the packaging signal, but in general this reduces titers considerably. One effective solution that is now in common use is to build expression vectors for the viral packaging proteins, Gag and Gag-Pol, with optimized/different amino acid coding sequences (50,51). This has several advantages: elimination of homology to the packaging signal as discussed here, potentially higher levels of Gag-Pol expression, and elimination of the dependency of Gag-Pol expression on Rev expression in the packaging system.

E. Gene Expression and RNA Nuclear Export Enhancers

Lentiviral genomes carry a series of elements that, in the virus, ensure the orderly progression of the viral life cycle, but that complicate life for vector makers (22). These include the Tat-mediated control of transcription that renders the HIV LTR a poor promoter in its absence, and the existence of RNA nuclear export inhibitory sequences in *env* and *gag-pol* primary transcripts, which are counteracted by the interaction of the Rev protein with the RRE. This allows export of the messages for the structural proteins and the full-length viral genome to the cytoplasm. These issues are addressed in the ''third-generation'' vector systems (15–17,46,47,52,53).

The Tat dependence of the LTR has been avoided by substituting other promoters (usually the CMV IE1 promoter that is very active in 293 cells) at the 5′ end, while leaving in place those elements that are needed to allow the transcript to be reverse transcribed and integrated (tRNA primer site and R region, packaging signal) (see also Figs. 2 and 3). The Rev dependence has been manipulated by including the RRE in the vector genome, but positioning it so that it is excluded from the transcript of the desired transgene, as it was believed

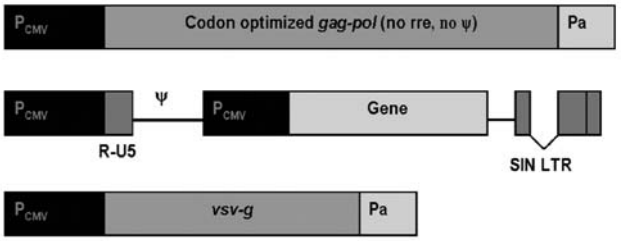

Figure 3 Components of a minimal vector system. The figure shows the 3 components necessary for Rev-independent packaging systems, such as with those designed with codon optimized *gag-pol* genes (50,51). The vector is also expressed without the need for Rev, and is shown in its plasmid configuration. When the transcribed RNA vector genome is packaged into a vector particle, then reverse transcribed and integrated into the genome of a target cell, the 5′CMV promoter disappears and is replaced by the SIN LTR, which has no promoter function. The encoded gene is expressed from the inner CMV promoter. The system shown is used for transient transfection. For a packaging cell line using the VSV-G protein as envelope, the gene encoding VSV-G would need to be inducible. P_{CMV}, CMV IE1 promoter. See the color insert for a color version of this figure.

to be desirable for high-level expression of the vector genomes. The transferred gene itself is expressed from an internal promoter. In most third-generation systems (Fig. 2), Rev is necessary anyway in the packaging cell to obtain good levels of expression of the Gag and Gag-Pol proteins. The packaging cell requirement for rev can be eliminated by the use of codon optimized *gag* and *gag-pol* genes, as noted above (50,51).

There has been benefit in some situations to add extra nucleic acid components to the vector genome to facilitate steps in the transduction cycle. The most commonly used elements include the cPPT element from the virus itself (see Section III.C) (30), which is part of the reverse transcriptase coding sequence and is a start site for viral genome replication (54,55); the Mason-Pfizer monkey virus constitutive transport element or analogs that facilitate export of unspliced RNA by interacting with a cellular activity (56); and the Woodchuck hepatitis virus posttranscriptional regulatory element element (57) that enhances the translation activity of the message in which it is incorporated by several possible mechanisms. All these elements can be shown to be quite potent in enhancing titer or potency of expression in some situations, but are also ineffective in others (e.g., 33). However, they have never been shown to be deleterious and so they or analogs are often included in the design of vectors without significant subsequent proof of their utility in the particular situation.

F. Envelope Proteins and Pseudotyping

The vast majority of lentiviral vectors have been made using the VSV-G pseudotype of the vector particles. This is for 2

main reasons: it works well in almost all vector systems to give high in vitro titer; and the vector produced can be easily concentrated up to about 1000-fold at the research scale, by centrifugation (11). This in turn yields preparations of vector that have titers of around 10^9 TU/mL, which turns out to be what is needed for a number of in vivo applications. However, numerous other pseudotypes (see Table 2 for some examples) have been made to try to provide tissue tropism, explore targetable envelopes, look for a nontoxic protein (as opposed to VSV-G) and hence simplify vector production, provide higher titers and also simply explore what novel properties such pseudotypes might possess. Although many such pseudotypes have been made, there is only preliminary information on the properties of such vector preparations in vivo, where it counts. An intriguing example has been the report of retrograde transport of the vector genome up the axons of neurons to the nucleus with rabies G pseudotyped vectors (58). This raises the possibility of introducing genes into the easily accessible muscle of a clinical subject and transducing the nuclei of neurons in the not-easily-accessible spinal cord, with prospects for treating motor neuron diseases.

Another aspect of the viral/vector life cycle that is beginning to be understood is the manner in which the viral capsid hijacks the endosomal transport pathways in order to travel to the inner side of the cell membrane. How envelope proteins gather there in the area of a coated pit to which the capsid arrives is less understood, as are the rules governing the subsets of the cellular surface proteins that gather there and bud off with the cellular membrane and the envelope protein (71). For example, HIV includes MHC class I and II molecules in the membrane of the particles (72). In MLV-based vectors, the amphotropic envelope probably allows inclusion of the CD55 and CD59 molecules that protect against complement attack in vivo, but VSV-G probably excludes them (73). The rules for such exclusion and inclusion of cellular markers are incompletely understood and have not been investigated in the context of the vectors or the different possible pseudotypes. One obvious consequence is that it is not known, for example, to what extent the vectors are resistant to degradation by human complement.

G. Other Cell and Tissue Tropism Issues

In vector systems, another classic way that does not involve envelope switches to achieve tissue-specific effects is to use a cell type-specific, or environment-specific, promoter and enhancer. This works quite well with lentiviral vectors (e.g., 74) (better, in fact, it seems than with MLV-type vectors), although there is not a large amount of data in vivo where, as noted before, it counts. The ease of accomplishment is related to the fact that ''SIN'' vectors (i.e., those with deletions in the 3′LTR that migrate to the 5′LTR after 1 round of reverse transcription and integration) (75) seem to work better with lentiviral vectors than with MLV-based vectors.

However, in addition to these intentional tissue-targeting issues there are innate tropisms in the vectors themselves that sometimes are unexpectedly observed. The best known of these is probably the failure of many HIV vectors to productively transduce some monkey cells (76). Further investigation shows that this phenomenon is quite common and can apply to nonprimate lentiviral vectors in primates (77). As primates are considered desirable models for human safety studies, and

Table 2 Examples of Various Pseudotypes of Lentiviral Vectors and Their Properties

Vector	Pseudotype	Observed properties	Reference
HIV	Amphotropic (A)-MLV	10-Fold lower titer than VSVg	9,12
FIV	A-MLV	10-Fold lower titer than VSV-G	47
EIAV	Rabies G	Retrograde transport in neuronal axons	58
HIV	Mokola	Titer less than VSV-G	59
HIV	Ebola, LCMV, mokola, A-MLV	Mokola has titer <VSV-G; ebola does not transduce CNS tissue	60
SIV, HIV	Sendai	May target liver cells	61,62
FIV	Alphavirus	Titers apparently better than VSV-G pseudotype	63
HIV	HTLV-1	Low titer ($<10^4$ TU/mL)	64
SIV	Mokola-G, A-MLV, influenza HA, LCMV	All preferentially transduce pigmented epithelial cells	65
HIV	HERV-W	Low titer ($<10^4$ TU/mL)	66
HIV	LCMV	Nontoxic in producer, titer equivalent to VSV-G, spin concentration possible	67
HIV	Avian leukosis-sarcoma virus	Low titer, removing cytoplasmic tail boosts titer	68
HIV	Ecotropic (E)-MLV, A-MLV	E-MLV pseudotype transduces mouse B cells <VSV-G	69
HIV	RD114-MLV, GALV-MLV Hybrids	Moderate titers (>A-MLV), spin concentration possible, resistant to complement	69,70

humans are the ultimate target population, this restriction of virus and vector entry could create problems. The phenomenon is likely akin to the N/B tropism of MLV-based vectors that maps to the capsid. The HIV/SIV restriction in some monkey cells also maps to the capsid (76), and it has recently been reported that the block can be overcome by substituting the cyclophilin A-binding region in the capsid with that from macrophage tropic HIV (78). Another example of unexpected restriction of transduction familiar to this author is that we were unable to transduce muscle tissue from mouse with FIV vectors but were able to easily introduce the same vectors into guinea pig muscle (47). However, it should also be noted that this restriction is generally saturatable, and, for example, HIV vectors have been used very successfully to treat a macaque model of Parkinson's disease (79), where there is a restriction to HIV entry.

Because the knowledge of the virology of these viruses is incomplete, we should expect further unforeseen observations.

IV. METHODS OF PRODUCTION AND TESTING

A. Viral Life Cycle

As for C-type viruses, the life cycle of lentiviruses can be conceptually divided into 2 stages for the purposes of using it as a vector. The entry, reverse transcription, transport into the nucleus and integration correspond to the use of the vector to carry heterologous genes into target cells. The subsequent provirus transcription synthesis of viral proteins, packaging of the viral genome, maturation of the viral particle, and export from the cell correspond to the production of the vector particles.

B. Transient and Stable Vector Producer Systems

There are 2 general categories of vector production procedures at present. These are transient transfection of highly transfectable cells (Fig. 3A), usually 293 or 293T cells with most or all the plasmids encoding the packaging and vector components (15–17,45–49), and production from stable cell lines already carrying some or all the necessary molecular components. The stable cell lines normally carry an inducible expression cassette for the VSV-G protein so that production of vector is triggered by induction of VSV-G expression (Fig. 3B). Because the VSV-G protein is toxic, these types of cells produce vector for at most around 4 days. This contrasts unfavorably with MLV-based packaging cells that use nontoxic envelopes and can produce vector for 2 weeks or more with 1 run.

No publications describe FIV packaging cell lines, although there are investigators working on these. There is 1 report about an early EIA V-based packaging cell (80). Several publications (81–85) have described HIV-1 packaging cell systems along these lines, but they have so far been put to very little use. This is probably the case for several reasons. First, these cell lines at present produce at best equivalent, more often lower, titers than the transient procedures (in the range of 10^5–10^6 TU/mL). Second, the packaging cell lines are more cumbersome to use in the research laboratory because it is likely that cellular clones of producer cell lines, carrying the vector of interest, would need to be isolated and tested, whereas the transient technique allows vector preparation to be rapidly generated. Third, packaging and producer lines usually would offer the prospect of continued harvest of vector for 2 weeks or more, but with inducible VSV-G, producer lines do not last more than a few days. Fourth, the real advantage of producer cell lines lies within the ability to characterize the vector-producing source in detail and this is of real advantage for late preclinical and clinical experiments, not for smaller lab or small animal experiments. It is not clear how much effort has gone into optimizing these systems, and there is certainly scope for some variation in approach. For example, it is known from MLV systems that limited induction of VSV-G is better than high levels (86). An SIV packaging cell line has also been described (87) and performs more or less like the HIV-based lines. In addition, cell lines for making true HIV vectors with HIV envelopes have been described and made in monkey cell lines (88–90), but the titers from these were 10e4/mL or less. One packaging cell line (82) was used in conjunction with a vector that initially is driven by a CMV promoter at the 5′ end, but after 1 round of replication the 5′ end acquires a tetracycline-inducible promoter (from the original 3′LTR). The attraction of this maneuver is that it allows transduction of vector genomes into the packaging cell, rather than transfection, but will also allow an effectively ''SIN'' configuration in target cells with an inactive (uninduced) promoter at the 5′LTR end. It is known from studies in MLV vectors that transduction of the vector, as opposed to transfection, into packaging cells allows higher vector genome expression (a limiting factor in making high titer producer lines) and clean insertion of multiple copies of the genome (91).

The components of the transient transfection system are those shown in Fig. 3a, or some variation thereof. In this situation, the expression of VSV-G does not have to be directed off an inducible promoter system as it is expressed transiently anyway. Because of the need for efficient transfection to make vector, almost all systems use the 293 or 293T (92) cell lines as the cell substrate. The major technical innovations here to make life easier have been the use of sodium butyrate to boost expression (93,94), and the use of standard transfection agents that are sold commercially (e.g., 95,96) to try and avoid the variability inherent in calcium phosphate precipitation methods. However, for clinical use it is likely that the calcium phosphate method will be preferred because, it seems that either most of the transfection agents have undefined components, the components are trade secret, and/or the manufacturer is unwilling to file a master file with the Food and Drug Administration (FDA).

C. Processing and Purification

The most common processing method by far for these vectors is high-speed centrifugation and resuspension (11). Done

once, this can concentrate the vectors about 1000-fold and the resulting preparations show little loss of activity and are surprisingly clean (i.e., the level of obvious contaminants from the tissue culture process such as BSA and DNA are not excessive) and with titers (see below) of around 10^9 TU/mL. This is, in fact, one of the reasons that the use of these vectors has become quite widespread. Such preparations are not toxic to animals in general, and the high potency allows administration to small animals such as mice and to tissues such as the brain and eye (see below), where it is impossible to effectively administer more than a few microliters. The resuspension buffer is typically ''isotonic'' PBS or Tris buffer with a cryoprotectant sugar and sometimes human serum albumin. The formulation of the vector preparations has not received much attention and investigators have usually extrapolated from useful retroviral vector formulations (91). There are no reports of stability studies.

It is possible to reconcentrate the 1000-fold concentrated material by further centrifugation, but beyond about 3000-fold total concentration results have not been good and difficulties in handling the material (viscosity etc.), toxicity, and significant losses begin to occur.

Alternate concentration methods are desirable because (1) centrifugation is a method that is notoriously difficult to scale up and use for manufacturing significant quantities of material for clinical trials and eventual product marketing; (2) alternate purification methods could be used with centrifugation to achieve higher concentrations; and (3) if other envelopes apart from VSV-G are to be used, it is likely that losses from centrifugation will be significant. There is a good deal of guidance for alternate procedures in the retroviral vector literature (e.g., 97) and column chromatography and other methods are starting to be used (98–100)

D. Titering

The titer or potency of vector preparations is typically measured by a dilution series of vector applied to target cells growing on tissue culture plates, allowing the cells to take up and express the vector, then stain for gene expression. This is descended from the classic phage plaque methods originally introduced by Max Delbruck and extended to mammalian viruses by Dulbecco (101). This type of measurement has served the viral and vector community well, but there are several issues with it. First, this works best with a marker that is easy to measure like beta-galactosidase or green fluorescence protein. Second, it is well known that this does not measure all the vector that is supplied to the plate in question, and that a similar ''titer'' can be observed by removing the applied supernatant and applying to a second plate. Third, along the same lines, it is possible to ''increase'' titers by methods such as ''spinoculation'' (102), where the cells are centrifuged during transduction. Fourth, the titer is very dependent on the cells used as target cells, the strain of the particular cell type, and seemingly minor changes in conditions. However, under highly standardized conditions this assay can be used to assess the potency of vector preparations.

For the reasons listed above, plus the fact that most vectors do not encode a gene product that is straightforward to detect in tissue culture, several other assays have been developed and used.

One common assay is the polymerase chain reaction (RT-PCR) measurement of the level of vector RNA in the preparation (103). In this assay, an aliquot of the vector preparation is disrupted and the level of some vector sequence (usually the packaging signal, although care must be taken to avoid competing gag sequences from the packaging construct) is measured by priming with oligomers for a first reverse transcription step followed by regular PCR amplification, usually followed in real time and measuring the number of PCR cycles necessary to observe a signal. Often this is compared in the same experiment to a beta-galactosidase vector preparation and a titer/mL obtained by comparing the two. Alternatively, an internally consistent measurement can be made against a known RNA standard. This assay is simple and rapid and samples the whole aliquot, so it is widely used. The drawbacks are that the relationship to actual in vitro and in vivo potency is not well characterized in general and this is probably vector dependent.

A third assay (the so-called ''DNA transduction'' assay) (96,103,104) is to perform the limiting dilution assay on tissue culture cells, as described above, then measure the level of the vector DNA as compared with cellular DNA directly by PCR (usually by the real-time, PCR cycle-to-detection method used for the RT-PCR assay). This in principle is an absolute measurement and is suitable as a potency assay, but uncertainties around the level of contamination and the rate of disappearance of DNA used in the transient transduction process, have to be dealt with. In addition, it is possible that all the lentiviral vector DNA may not integrate immediately (12), so the timing of the measurement may make a difference.

A fourth assay used with HIV-based vectors is simply to measure the levels of the p24 capsid protein (e.g., 35) using inexpensive widely available commercial kits (used for blood testing). This assay is very robust but measures only the level of p24 in the preparation. The link between this number and actual transduction potency is not obvious, and may be different with different vector preparations. Such assays form part of the basic characterization of vector preparations, but it is recognized that, at present, these preparations have properties that are not necessarily consistent from batch to batch. Such inconsistency may be linked to issues such as the ratio of active to inactive particles, the extent of encapsidation of nonviral RNA, the cell line from which the particles were made, and the variations in the preparation methods in general. Further characterization of these issues is being undertaken and will be necessary as these vectors become more commonly used in the clinic. (See, for example, ''Guidance for Human Somatic Cell and Gene Therapy,'' U.S. Department of Health and Human Services, FDA, Center for Biologics Evaluation and Research, March 1998, http://www.fda.gov/cber/gdlns/somgene.pdf 1998.)

E. Replication-competent Lentivirus and Other Safety Issues in Manufacturing

Biological entities, in terms of FDA definitions of product types, are defined by the process by which they are made, such that robust, reliable manufacturing processes will need to be developed further. However, an important issue that has attracted a good deal of attention is the possibility of regenerating some form of replicating lentiviral entity. This attention was engendered by both the precedent of replication-competent retrovirus being generated in MLV-type production systems and its link to lymphoma in monkeys (5), and the fact that HIV is a well-known and potentially lethal human pathogen. This issue was one force driving the development of the stripped-down, accessory protein-minimized, third-generation production systems. It is likely that these efforts have made generation of replication-competent lentivirus (RCL) (105) in third-generation systems, in the manner seen for MLV type systems, close to impossible, and that any replicating agent may need to use or acquire by recombination, cellular components as well as the viral system machinery. For example, an envelope from a human endogenous retrovirus can pseudotype HIV-1 (66), when the gene is supplied in trans. This potential acquisition of cellular components through some form of nonhomologous recombination will likely need to be even more extensive to generate a hypothetical replicating entity from cells making vectors based on viruses such as FIV and EIAV that are not capable of replication in human cells. Nevertheless this issue is perceived as significant and the likelihood of generating RCI will be judged by the data generated in vector production and testing. Therefore, tests for RCL form a key element of testing of vector preparations. The major difficulty is that, as it is unclear exactly what the properties of the replicating entity would be, designing a reliable test and spike control has been difficult. In general, a putative RCL needs to be able to replicate in amplifier or test cells to be detected. It has been agreed generally that the risk involved to construct, test, and use experimentally some of the postulated hybrids outweighs any possible general benefit to testing of vector preparations. For example, a version of HIV without accessory proteins and a VSV-G type envelope could have extremely unpredictable properties if a human infection occurred. No investigator wants to use this.

Therefore, various test methods have been proposed and developed. These include the use of cell lines that express an envelope and the use of an HIV *gag-pol* vector as the spike with read-out being the production of p24 (84), as well as the use of the ''product-enhanced reverse transcriptase assay'', which assays for the RT activity on a defined template (96), and then amplifies the defined RT product by PCR and detects using real-time PCR cycle-dependent assay. The spike in this case could be almost any replicating retrovirus such as MLV. Both these assays require some cellular passaging to get rid of vector signal and hopefully amplify or conserve the RCL signal. Alternative assays look directly for postulated recombination products by PCR but have the drawbacks that this represents a guess at what the entity would be and also it does not actually test for replicating agents.

V. TARGET TISSUES AND THERAPEUTIC MODELS

Gene therapy techniques, as currently applied to the treatment of disease, tend to develop as follows. A vector is made, tried out in tissue culture and small animal models to see how it performs in terms of tissue tropism, the results assessed and a disease process identified that might be susceptible to modification. This is a perfectly rational way to proceed given the complexity of the technology and disease pathogenesis, but it has the disadvantage that the clinical element arrives late and the choice of clinical target has not always been well made. One of the exciting aspects about lentiviral vectors has been the early clear identification of tissues and disease areas where the technology seems to have great potential and there are large clinical needs. Time will tell if the development of these vectors will, in fact, lead to clinically useful therapies, but there is no doubt about the promise.

One of the first areas identified as a target was the brain. Injection of small amounts of high titer material leads to localized long-lived expression of the encoded genes (12,46,106–111c) (Fig. 4.). This has a seemingly natural fit with the supposed need for chronic therapies for chronic neurodegenerative diseases. The VSV-G pseudotyped material from HIV, FIV, and EIAV vectors have been shown to efficiently transduce neurons in various areas of the brain and express the transgene(s) for as long as anyone has looked so far. Such experiments have been performed in mice, rats, and monkeys. Thus, animal model data for the treatment of lysosomal storage diseases (112,113), Huntington's disease (114), Parkinson's disease (79,115,116), and other diseases have been generated. One prominent example has been the recovery from a model of Parkinson's disease in primates (79) after administration of an HIV vector encoding glial cell derived nerve growth factor. It is hard to see how a protein can be delivered chronically to large numbers of people, as may be needed, without some kind of gene therapy. The flip side of this is that, of course, it is necessary have some control over expression of a growth factor in the brain, and this technology (e.g., 117–120) will also need to be clinically developed. Nevertheless, the results are encouraging enough that several groups are starting to undertake this.

A related area that shows promise is the delivery of genes to the eye for treatment of various types of retinopathies, including macular degeneration and diabetic retinopathy (121–125). This interest springs, largely, from the observation that lentiviral vectors seem to have particular tropism for pigmented retinal epithelial cells in several species. Most proposals center on preventing the disorganized angiogenesis that follows disease onset and try to prevent it as it exacerbates clinical symptoms. There is currently a clinical trial underway using adenoviral vectors encoding the angiogenesis inhibitor pigment derived epithelium factor (126), but presumably len-

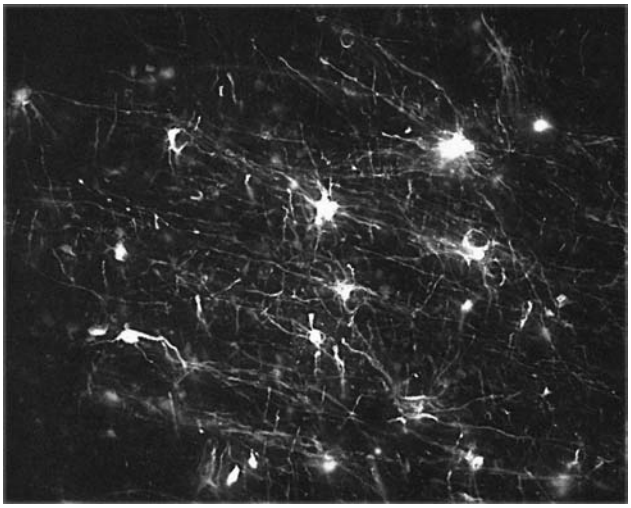

Figure 4 Efficient transduction of neural tissue in vivo with a lentiviral vector. The photomicrograph shows GFP expression in the striatum of a squirrel monkey at 18 days following infusion of 7.5 uL of an EIAV-based VSV-G pseudotyped vector [pSMART(1)G]) encoding GFP (2.4×10^{10} TU/mL) into the right caudate nucleus. Lentiviral vector was infused at a rate of 0.5 uL/min. Extensive expression of GFP can be seen throughout both cell bodies and processes. Cells morphologically similar to both neurons and glia expressed high levels of GFP. See the color insert for a color version of this figure.

tiviral vectors may be a better fit. Once again, however, clinically it would be desirable to be able to switch the gene off. If a VSV-G lentiviral vector is injected intravenously in a mouse, it localizes mainly to the liver, spleen, and bone marrow (74,127,128). The levels decline somewhat over time in the liver and spleen but may stay at the initial level in the marrow. These levels are not completely consistent but are in the 0.1% to 30% range of total cell sampled. This observation points to some other promising target tissues, namely, liver and bone marrow. The spleen observation raised the possibility of inducing immune responses and has not attracted as much attention, except in vaccine-oriented applications.

The ability of lentiviral vectors to transduce liver tissue in mice and rats has been the subject of some debate, and there are definitely some issues that are not fully understood. Some investigators see the need to make the liver cells at least be part of a regenerating or proliferating tissue (34,129,130), whereas others experience no problem in generating therapeutic levels of systemic protein after liver targeted transduction (35,74,131). It seems likely that such differences are due to subtle vector differences or precise details of the delivery method, which have not yet been completely identified. It does seem that explanted primary hepatocytes that are not replicating are efficiently transduced (132), and pseudotyping with Sendai virus envelope has been used to try to target liver cells (61). The indications that are most discussed here are treatments that require systemic protein over long periods of time such as hemophilia A and B.

There is little investigation of transducing bone marrow in vivo but a lot of effort has been devoted to using the vectors to deliver genes to hematopoietic stem cells (HSCs). This is because the transplantation technology is in clinical use and the lentiviral vectors offer the opportunity to transduce the cells in vitro before reimplantation, without the need to make these cells pass through the cell cycle, as has been required for MLV-based vectors. This has been performed in mouse models and in human HSC in immune-deficient mouse models (133,134). Targets here include genetic diseases such as thalassemia (135,136), sickle-cell anemia (137), and chronic granulomatous disease (138). In addition, anti-HIV therapy has been investigated to provide blood cells derived from HSC that are protected from HIV or simply transducing T cells directly (139). Among the anti-HIV strategies are rescuable anti-HIV genes (140), antisense (141), and iRNA (142) to constitutively protect cells (143).

Because of the characteristics of the vector, there has only been a small amount of activity in cancer (e.g., 144,145), a target that has attracted a lot of attention from other vector systems. In addition, there have been attempts to use the vectors for lung application, such as cystic fibrosis (146), where some understanding of the issues around tranducing the apical side of the lung epithelial cells has been developed. Other targets include collagen deficiency (147) and kidney disease (148).

VI. CLINICAL AND CLINICAL SAFETY ISSUES

As this is written, these vectors appear to be on the point of being used in the clinic (see http://www.virxsys.com/PressReleaseFINAL20030213.pdf). The first indication is likely to be for HIV therapy, but it is likely that protocols for neurodegenerative diseases such as Parkinson's disease will follow. In the clinic, the major vector-related safety issues [apart from contamination of vector preparations with adventitious agents (149) such as RCL, and see above) that have been raised relate to recombination or rescue by preexisting (and perhaps unrecognized) infection with HIV (150–152), interaction with endogenous sequences (65) in the trial subject, and more recently insertional mutagenesis (4,153,154) and resultant predilection to development of cancers. The HIV ''rescue'' issue can probably be dealt with by using SIN vectors (75) that are not able to be rescued, although the final answer will come from clinical data. It should also be noted that large numbers of HIV-infected individuals have been treated with MLV vectors without obvious vector-related side effects [see the web site of the National Institutes of Health Office of Biotechnology Activities (OBA) at http://www4.od.nih.gov/oba/rac/SAE_rpts/Mod0902s/Sep02_MODs.htm]. The insertional mutagenesis issue has been prompted by the appearance of treatment-related leukemias in 2 of 11 children treated by bone

marrow transduction and reimplantation (153,154) from X-linked severe combined immunodeficiency disease (X-SCID). The data in the first child suggests some kind of transcriptional activation event of the LMO-2 gene was involved. Interestingly, it has been reported that lentiviral vectors should be less likely to have this kind of effect as they have less read through of the 3′LTR polyadenylation site than vectors based on MLV-type C retrovirus (155). However, lentiviral SIN vectors are as leaky as MLV vectors. The X-SCID trial was performed using MLV-type vectors, and the results were unexpected given the large numbers of other retroviral vector-treated trial patients in unrelated protocols. Nevertheless, the observation will undoubtedly lead to further examination of the probability of such events in other trials using integrating vectors (155). There is no indication, however, from the hundreds or thousands of animals treated with lentiviral vectors so far that this issue will be a significant one in general.

VII. SUMMARY

Lentiviral vector development is at an exciting stage. Groundwork sufficient to allow their clinical use has been laid, although much more remains to be done before such agents become part of a licensed drug. The areas where such agents could be useful have been identified, and it now remains to see how predictive the extensive research and preclinical data are for human patients. Experience suggests that success in the preliminary indications will lead to further areas of use.

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8

Ex Vivo Gene Therapy Using Myoblasts and Regulatable Retroviral Vectors

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I. INTRODUCTION: SKELETAL MUSCLE AS A TARGET TISSUE FOR GENE THERAPY

Human gene therapy—clinical treatments aimed at introducing or repairing genes to provide long-term correction for a defect caused by acquired or inherited disease—has been rapidly shuttled from a realm of theory and speculation into one of impending reality. The first approved protocol for human somatic gene therapy entered clinical trials in 1990 (1); the number of clinical trials worldwide has since burgeoned into the hundreds. Although gene therapy is still in its infancy and has yet to overcome a variety of pitfalls and problems, significant progress has been made. In this chapter, we discuss some advances in developing efficient means of gene delivery and in regulating gene expression to achieve pulsatile protein delivery when desired and avoid toxic levels. Although many of the features of muscle described below make it ideally suited for adenoviral, adeno-associated viral (AAV), lentiviral, and naked DNA delivery, the primary focus of this is ex vivo gene delivery via intramuscular cell implantation. Ex vivo myoblast-mediated gene delivery results in robust, long-term production in skeletal muscle of recombinant proteins ranging from muscle-specific proteins to systemic circulatory factors. This approach holds the advantage over direct viral or DNA delivery that the genetic change occurs outside the body, and the transgenic cells can be screened for potentially deleterious consequences like tumorigenicity before being implanted into a patient. Moreover, problematic immunological effects currently associated with most other methods are avoided with autologous myoblast-mediated delivery.

Of the many preclinical studies past and present, a number of approaches have employed skeletal muscle for delivery of genes in attempts to treat muscle disorders and other types of diseases. Skeletal muscle has been a target of special interest for gene therapy because of inherent properties that set it apart from other tissue types. Among its advantages include the fact that it is a very well-studied and understood tissue. This knowledge is invaluable for engineering strategies of gene delivery, and for assessing and controlling therapeutic protein expression. Skeletal muscle comprises a large percentage of the total body mass and thus is easily accessible to gene delivery. Mature, differentiated myofibers are relatively long lived, providing a lasting substrate for the stable expression of recombinant genes. Myoblasts that are purified and genetically engineered in tissue culture can be reinjected and will then enter host fibers where they are nurtured, properly innervated, and in close proximity to the blood. The multinucleate nature of muscle cells facilitates delivery of 2 or more different vectors that encode products that can meet inside the cell. This is particularly advantageous for vectors with limited capacity, such as AAV, or for introducing gene regulatory systems (see below). Moreover, although rich in contractile apparatus and not obviously suited for secretion, genetically altered skeletal muscle tissue has proven to be a surprisingly efficient factory for the production and delivery of recombinant proteins to the circulation, allowing for the treatment of a broad array of muscle and nonmuscle disorders where cell type-specific expression is not required.

In addition to the ex vivo gene transfer approach, an in vivo method of gene delivery to muscle is currently being tested and developed. In the in vivo approach, a vector harboring a copy of a corrected gene or encoding a product that can remedy a patient's defect is introduced directly into the muscle tissue of the patient. The vector employed may be either viral

or nonviral in nature. Viral vectors that have been examined for their ability to transduce nondividing cells characteristic of muscle tissues include adenovirus, AAV, lentivirus, and herpes simplex type-1 virus (2–4). Although promising, in vivo approaches using viral vectors face several challenges, such as immune reactions elicited against viral elements (2,3). Although the most persistent expression has been seen with AAV (5–9), small capacity limits cDNA size, and difficulties in achieving adequate viral titers necessary for clinical trials have yet to be overcome. New generations of viral vectors characterized by more efficient production, lower immunogenicity and more stable expression are currently being developed that may prove to be powerful tools for muscle-mediated gene therapy.

Nonviral in vivo approaches for gene delivery to skeletal muscle center on intramuscular injection of plasmid DNA vectors. In the early 1990s, studies by Wolff and coworkers demonstrated that direct injection of naked plasmid DNA directly into the muscle tissues of mice led to transfection of skeletal myocytes and persistence of expression for at least 19 months in vivo (10,11). Naked plasmid DNA has also been shown to be taken up and expressed by cardiac muscle and skin (12–15). Plasmid vectors have a number of advantages for muscle-based gene therapy, and efforts to improve the efficiency of delivery appear warranted. These include simplicity of preparation and introduction into the host, and ability to be produced and stored in large quantities. In addition, the vectors are nonviral and are unlikely to be transmitted to other tissues. They also do not integrate into the genomes of host cells, precluding the risk of cancer by activation of a neighboring oncogene. Because achieving high levels of transgene expression using this approach has been problematic, direct plasmid DNA injection has been applied mostly to applications where only very low levels of transgene expression are required. One such application is in using intramuscular injection of plasmid DNA for vaccination purposes (16–19). Plasmids encoding antigenic proteins may be used to generate host antibodies specific to the antigen; because only small amounts of antigen are needed to elicit an immune response, plasmid vectors are well suited for this purpose. Recently, new progress has been made in developing methods for attaining stable, high-level gene expression using plasmid vectors. These include modifying DNA sequences within plasmids to enhance transcriptional efficiency of the vector (20), and combining plasmid DNA injection with the delivery of electric pulses to increase efficiency of myofiber transfection (21,22). Although promising, these methods require further study and development before they can be effectively applied to the therapeutic realm.

Cell-mediated or ex vivo gene delivery may provide a method of drug delivery for the treatment of a wide range of diseases (23). Skeletal muscle cells can be maintained as either proliferating or differentiating cells. The proliferative cells, known as myoblasts, are mononucleate muscle progenitors capable of fusing with each other to form new muscle fibers, or with preexisting myofibers (Fig. 1A). Myoblasts can be readily isolated from muscle and expanded in cell culture.

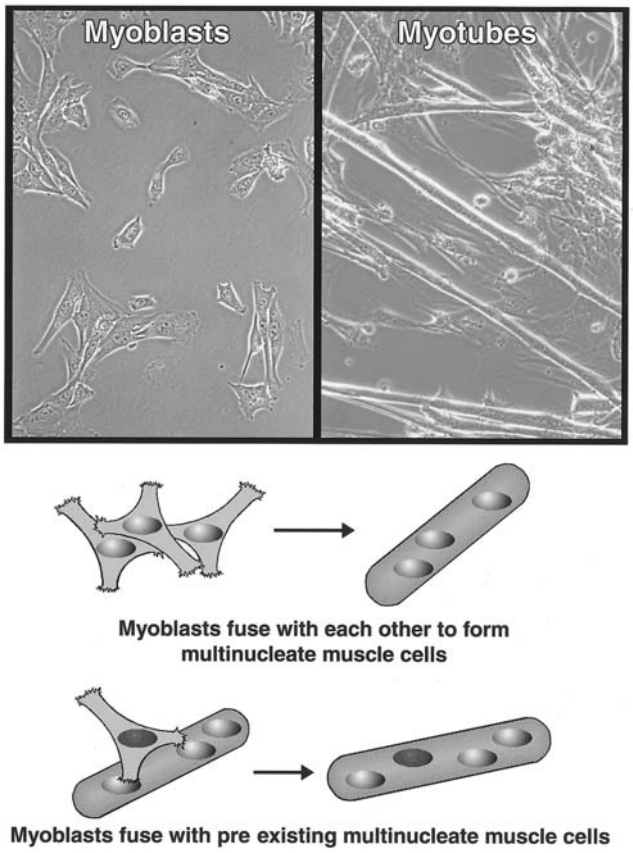

Figure 1 Myoblasts fuse to form multinucleate muscle cells. (A) Myoblasts are mononucleate muscle progenitor cells that can be isolated from muscle tissue and grown in cell culture (left). When provided with appropriate growth conditions, myoblasts fuse with each other in culture to form long, cylindrical, multinucleate myotubes (right). (B) Myoblasts can either fuse with each other to form new muscle cells, or they can fuse with preexisting muscle cells. Myoblasts of one genotype (shown with a dark gray nucleus) can fuse with multinucleate muscle cells of another genotype (shown with a light gray nucleus), thus delivering new genetic information to the preexisting muscle cell.

In vitro, they may be genetically engineered and extensively characterized, and then reimplanted back into muscle, where they stably fuse with myofibers (Fig. 1B). This unique property of skeletal muscle tissue has allowed for the development of myoblast-mediated gene transfer (24–26). Although the ex vivo approach of gene transfer to muscle is currently more cumbersome and costly than the in vivo approach, it provides certain advantages not offered by in vivo methods. Genetically altered myoblasts may be fully characterized in vitro before in vivo injection to ensure secretion of recombinant products of correct size and function at physiologically useful levels. In addition, isolated myoblasts are engineered outside the

body with retroviruses, a process that generally ensures that only the proper cell type is transduced. In contrast, introduction of viral or nonviral vectors directly into muscle could theoretically lead to inadvertent low-level transduction of cells other than those being targeted, for example cells of the germline. Finally, recent studies suggest that a major limitation to ex vivo gene delivery—the requirement that syngeneic myoblasts isolated from 1 patient be reinjected into that same patient to avoid rejection of cells by the immune system—may be overcome. Encapsulation of myoblasts overrides the requirement for a ''tailor-made'' therapy, allowing allogeneic cells that are invisible to the immune system to be used. Myoblasts appear advantageous over other cell types for this purpose because they do not overgrow and die within capsules, but instead differentiate and persist (27–29). Although this procedure prevents myoblasts from fusing into preexisting muscle, it theoretically allows universal donor cells to be derived from muscles of a single patient and implanted at ectopic sites in different patients for delivery of diverse products. All these advantages make ex vivo gene delivery via myoblasts a promising candidate for human gene therapy in the future. The remainder of this chapter focuses on ex vivo gene delivery to skeletal muscle.

II. DEVELOPMENT OF EX VIVO GENE DELIVERY BY MYOBLAST TRANSPLANTATION

A. Evidence for Myogenic Precursor Cells: Myoblasts

Skeletal muscle comprises approximately 10% of the total human body mass and is highly accessible to manipulation. A typical striated mature skeletal muscle cell, known as a myofiber, is large (1–40 mm in length and 10–50 microns wide), cylindrical, and multinucleated (as many as 100 nuclei per cell). Myofibers contain many nuclei because they are formed during development by the fusion of mononucleated precursor cells known as myoblasts. Myoblasts persist in mature muscle tissue as satellite cells, which can be viewed by electron microscopy as being ''wedged'' between the plasma membrane of the myofiber and the surrounding extracellular matrix (30) (Fig. 2). These cells can continue to fuse to neighboring myofibers in mature muscle, aiding in new muscle formation during regeneration following injury (31).

Interest in myoblasts as vehicles for gene delivery arose from studies of muscle cell biology and development. Studies of pattern formation in skeletal muscle showed that myoblasts are greatly influenced by extrinsic factors and become integrated into existing muscle fibers. Mammalian skeletal muscle is composed of a complex pattern of myofibers. Fiber types differ in their rate of contraction (fast and slow), determined in part by the ratio of fast and slow myosin heavy chain (MyHC) isoforms contained in each fiber (32). Although both fiber types occur in all skeletal muscles, the ratio of the 2 classes differs between muscles and even between different regions of a single muscle (33). Whereas lineage and myoblast-intrinsic

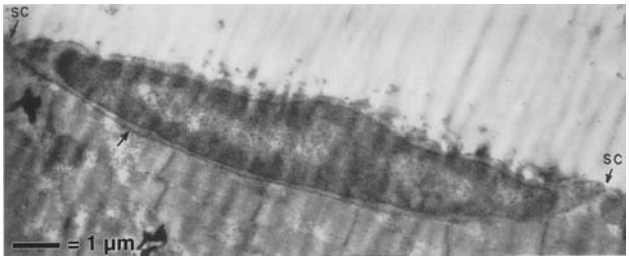

Figure 2 Satellite cell viewed by electron microscopy. Electron micrograph of a satellite cell in frog skeletal muscle, seen in longitudinal view. Extreme poles of the cell are indicated (sc). The arrow marks where the plasma membrane of the satellite cell juxtaposes that of the muscle fiber. (Reproduced from Ref. 30, p. 495, by copyright permission of The Rockefeller University Press.)

properties play a role in muscle fiber patterning, as shown by the finding that myoblasts expressing different slow isoforms are characteristic of different developmental stages (34), a number of experiments suggest that the environment is important in the generation and maintenance of that pattern (35). During early development of human limb muscle when multiple fiber types are forming, virtually all myoblasts, irrespective of the stage of development from which they are taken, give rise to clones expressing slow MyHC upon differentiation in culture. This is seen to be true even when myoblasts are taken from muscle at midgestation, when only 3% of fibers in vivo express slow MyHC. These results suggest that although culture conditions allow for slow MyHC expression in myoblasts, such expression seems to be repressed by extrinsic factors in vivo.

Additional experiments using retroviruses as heritable markers of cell fate in vivo further solidified these findings. When retroviral vectors encoding the reporter gene lacZ were injected directly into muscles of postnatal rats, clusters of multiply labeled fibers arising from progeny of single satellite cells were observed (24). The basal lamina, a connective sheath surrounding each muscle fiber, did not appear to prevent migration of labeled myoblasts into multiple fibers. Moreover, rat myoblast clones were shown to contribute progeny to both slow and fast muscle fiber types in their vicinity in vivo (25). These results demonstrate that mammalian myoblasts fuse randomly with all fiber types encountered, and adopt the pattern of myogenic gene expression characteristic of the host muscle fiber.

Transplantation studies further developed the notion of employing myoblasts for gene delivery, using allografts of minced muscle tissue (36) or pieces of intact muscle (37), and also by injection of muscle precursor cells (38–41). In these studies, isoforms of the enzyme glucose-6-phosphate isomerase were employed as markers to differentiate contributions of donor and host myoblasts to myofibers. The detection of hybrid fibers expressing isoforms containing subunits de-

rived from donor- and host-provided evidence that grafts of muscle precursor cells could alter the genetic makeup of, and contribute muscle proteins to, mature myofibers.

These initial experiments were of importance in establishing that myoblasts can fuse with all muscle fiber types in their vicinity, becoming fully integrated into mature muscle tissue that has access to the circulation and is innervated. In addition, muscle precursor cells of one genotype that are injected into muscle tissue of another genotype fuse to form hybrid muscle fibers, where they are capable of expressing donor genes. These findings paved the way for later studies examining applications of myoblast transplantation for the correction of various diseases.

B. Methodology: Purification, Growth, and Transduction of Primary Myoblasts

The ease of isolating myoblasts from both mouse and human muscle, and purifying, growing, and transducing them in vitro is a major advantage of using myoblasts rather than other cell types for gene transfer. Primary myoblasts can be isolated from any mouse strain—including strains carrying genetic mutations or transgenic strains (42,43)—providing a broad array of genotypes either for study in tissue culture or for transplantation. Moreover, because myoblasts may be isolated from a specific donor for implantation into a syngeneic host, problems of immunoincompatibility are obviated. Although established myoblast cell lines, such as the C2 myogenic cell line, may also be used for implantation, these cells can proliferate and form tumors when implanted into mice (44). In contrast, despite their impressive capacity to proliferate in culture, primary muscle cells do not form tumors upon injection into mouse muscle (44), and in the case of human myoblasts exhibit Hayflick-like senescence but not transformation (45).

Myoblasts can be isolated from muscle tissues from individuals of all ages, although both the yield and number of doublings tend to be higher if obtained from younger donors. Primary cultures are derived from postnatal muscle using mechanical or enzymatic dissociation methods (44,46), and readily obtained from human biopsy or autopsy tissue (47). Because such cultures are composed of a mixture of cell types, the population of myoblasts must be further purified. For primary cultures isolated from mice, this is accomplished using cell culture conditions that favor myoblast growth at the expense of other cell types, such that a pure population of myoblasts can be obtained within 2 weeks of normal growth (26,44). Human myoblasts can be purified by sorting in a fluorescence-activated cell sorter (FACS), employing fluorescent antibodies specific to the muscle surface antigen H31 or neural cell adhesion molecule (NCAM) (46). The cells that are isolated are capable of self-renewal and can undergo at least 40 cell doublings without differentiating (46). This implies that a kilogram of cells for transplantation use may be derived from a 5-mm^3 biopsy. Mouse (48) and rat (49) primary cells have also been isolated by FACS using antibodies to α7 integrin. Thus, a large population of rodent or human myoblasts may be easily obtained, purified, and expanded in cell culture.

Recombinant genes can be stably introduced into isolated and purified myoblasts using a number of methods, including lipofection, calcium-mediated transfection, or (more readily) by retroviral infection. Using conditions optimized for retroviral infection of myoblasts at high efficiency, 99% of primary myoblasts in culture are easily transduced without use of a selectable marker (50). This enables the creation of pure populations of primary myoblasts expressing a gene of interest, free of contamination by most nonexpressing cells. Myoblasts do not lose their ability to mature and differentiate by the process of being genetically altered.

III. APPLICATIONS OF MYOBLAST-MEDIATED EX VIVO GENE DELIVERY

Primary mouse myoblasts stably express recombinant genes following transduction. Myoblasts retrovirally transduced with the bacterial *lacZ* gene and injected into mouse skeletal muscle fuse with muscle fibers and express high levels of β-galactosidase (Fig. 3). The β-galactosidase expression can be observed in hybrid myofibers for at least 6 months (44). Other studies have shown that stable levels of recombinant proteins are produced for at least 10 months (51). Myoblasts that have been genetically engineered to express a recombinant gene may thus be used for stable delivery of that gene into the body (Fig. 4).

Although not widely viewed as a secretory tissue, skeletal muscle is highly vascularized and recombinant proteins secreted from myoblasts readily gain access to circulation. Initial studies using C2C12 myoblasts genetically altered to express human growth hormone (hGH) first demonstrated this to be the case (52,53). hGH was chosen as the gene of interest because it has a very short half-life in mouse serum (4 min) (54), providing a stringent test for sustained production and secretion into the circulation over time. After injection of genetically engineered myoblasts into mouse muscle, stable physiological levels of hGH could be detected for at least 3 months (Fig. 5). These results showed that myoblasts, by fusing with preexisting multinucleated myofibers, can serve as vehicles for systemic delivery of recombinant proteins. Thus, skeletal muscle may be used as a factory for production of a range of secreted gene products for treatment of nonmuscle-related disorders. Because muscle is capable of carrying out posttranslational modifications normally performed by other tissues (e.g., gamma carboxylation essential for production of functional coagulation factors in the liver), such recombinant nonmuscle proteins are biologically active even when produced by muscle (55–58). Applications of myoblast-mediated gene delivery to treat diseases affecting both muscle and other tissues are discussed in the following section.

IV. DISEASE TARGETS FOR MYOBLAST-MEDIATED GENE TRANSFER

A. Muscular Dystrophies

The concept of applying myoblast transplantation to the treatment of disease was a natural outcome of the many studies

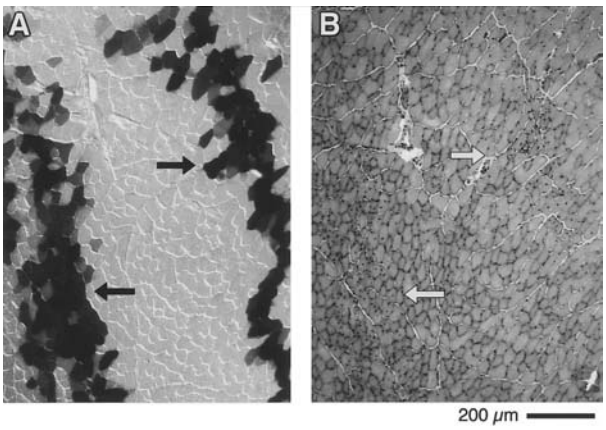

Figure 3 Incorporation of β-galactosidase-expressing myoblasts into skeletal muscle. Primary mouse myoblasts transduced with the reporter gene lacZ, encoding the bacterial β-galactosidase enzyme, were injected into mouse leg skeletal muscle, where they formed hybrid myofibers with host muscle. Injected muscles were isolated and frozen, and cryostat sections were prepared for histological analysis. (A) Hybrid myofibers producing β-galactosidase at the implantation sites can be seen as dark fibers after staining with the enzyme's substrate X-gal. (B) An adjacent section was stained with hematoxylin/eosin to show tissue architecture, and demonstrates that the hybrid fibers are of normal diameter and morphology and are an integral part of the muscle tissue. The centrally located nuclei that can be observed in (B) are indicative of myofibers that have undergone regeneration, and represent a normal response to a needle injection. The arrows denote corresponding regions in the two sections.

establishing myoblasts as potent vehicles for delivering donor genes into host muscle. The first approaches centered on the use of allografts of normal precursor cells to insert donor nuclei, containing a normal genome, into genetically abnormal muscle. Although not technically gene therapy (because donor myoblasts were not genetically engineered in any way), such ''cell therapy'' experiments were important in establishing the utility of myoblast-mediated gene delivery for the treatment of disease, and are the only studies involving myoblast transplantation that have translated into human clinical trials to date. The first disorder to which this therapeutic approach was applied was Duchenne muscular dystrophy (DMD), the most common of heritable human muscular dystrophies. DMD affects 1 in 3000 males and causes progressive muscle weakness beginning in childhood; patients with severe forms rarely survive past early adulthood. DMD is caused by mutations in the gene *dystrophin* (59), a large gene encoding a structural protein involved in anchoring skeletal myofibers to the extracellular matrix. By implanting myoblasts that contained normal copies of the *dystrophin* gene into dystrophin-deficient muscle, researchers hoped to rescue the genetic defect in humans as previously achieved in mdx mice, the mouse model of disease (41). In mdx mice, the implanted myoblasts were able to render host myofibers dystrophin positive while counteracting the characteristic cycle of fiber degeneration and regeneration characteristic of mdx muscle (41,60).

Clinical trials in which donor myoblasts taken from normal human muscle were introduced into DMD patients were initiated at multiple institutions (61–67). All these studies demonstrated that myoblast implantation into humans has no adverse effects. However, all but 1 group reported the disappointing finding that only a very small percentage of host myofibers resulted in normal dystrophin expression. At the protein level, these results could have been due to reversion or occasional expression by mutant host fibers of a truncated dystrophin detectable by antibodies. One group, however, provided definitive evidence that donor dystrophin transcripts were being synthesized by polymerase chain reaction (PCR) (62). Experiments combining fluorescent in situ hybridization (FISH) together with immunohistochemistry were recently conducted to examine the fate of individual myoblasts after implantation into muscles of DMD patients (68,69). This combination of techniques allowed the localization of both the dystrophin protein and the donor nuclei themselves, permitting more quantitative assessment of the efficiency of myoblast transfer. Findings from these studies showed that a large proportion of donor myoblasts successfully integrated into host myofibers in almost every subject; donor nuclei were interspersed with and aligned with host nuclei. Furthermore, these experiments demonstrated that increased dystrophin expression observed in recipient muscle was contributed by the donor nuclei and was not due to spontaneous reversion of the mutated *dystrophin* gene because the antibodies used were specific to the product for the deleted gene regions in the recipient. Moreover, the dystrophin produced by single nuclei spanned regions, including 20 to 30 nuclei. Why only a subset of transduced myofibers expressed dystrophin is still not understood. One hypothesis is that variables related to the DMD disease state itself, such as increased fibrosis with patient age, impaired myoblast access. An alternative hypothesis is that nuclei were not transcriptionally active in regions of fibers undergoing degeneration.

For treatment of muscular dystrophies by gene therapy, a large proportion of muscle fibers must be transduced to produce a beneficial outcome. Furthermore, myoblasts must be implanted into all muscles, some of which are difficult to access, such as the diaphragm and heart. Failure of these latter muscles is the cause of death in patients with DMD. These represent major challenges to myoblast-mediated gene delivery in treating inherited myopathies and suggest that a cell-based method may not be practical. Histochemical staining and enzymatic activity assays of muscle transplanted with β-galactosidase-expressing myoblasts show that the total number of labeled fibers and the total β-galactosidase activity is maximal at the implantation site, and decreases in parallel with increasing distance from the site (70). Although myoblasts were believed to be able to migrate from the circulation to damaged muscle (71), this is certainly not a frequent event. Thus, to target a high percentage of myofibers in multiple

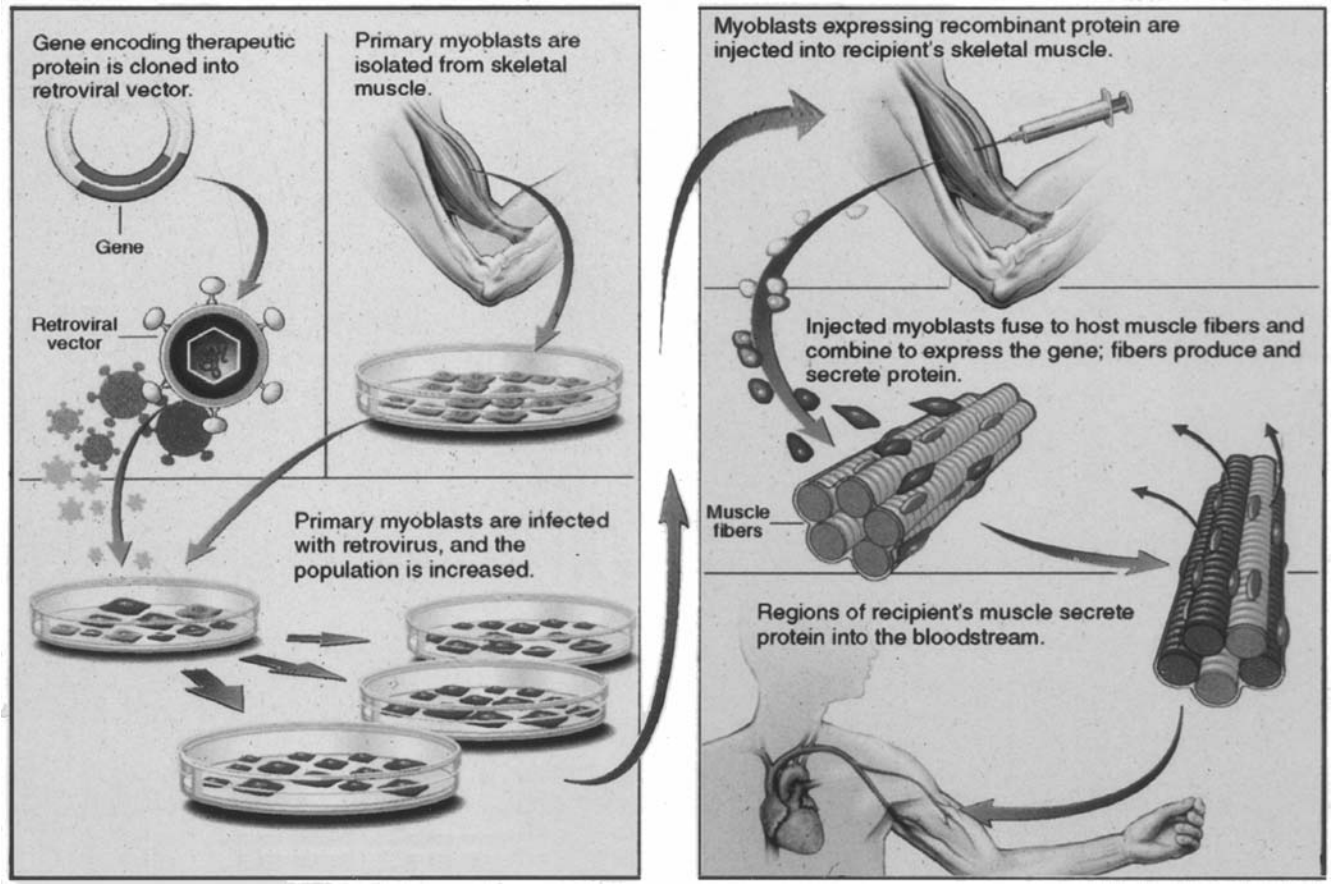

Figure 4 Muscle-mediated gene therapy. Muscle-mediated gene therapy by implantation of genetically engineered myoblasts allows for delivery of diverse therapeutic proteins, either directly to muscle or (as shown) to the systemic circulation. (Adapted with permission from Ref. 23, p. 1555. Copyright © 1995 Massachusetts Medical Society. All rights reserved.)

muscles of large organisms such as humans, delivery of viral vectors and naked DNA encoding either full-length or truncated dystrophin genes (72–74), or in the future the ubiquitous utrophin (75), may be most effective.

B. Lysosomal Storage Diseases and Serum Protein Deficiencies: Treatment by Secreted Circulating Recombinant Proteins

As described above, studies with hGH showed that myofibers efficiently secrete recombinant proteins that readily gain access to the circulation. Myoblast-mediated gene transfer has been further employed to express therapeutic proteins not normally made by muscle (51–53,55–58,76–79). Here we describe in further detail progress made in studies in which genes encoding β-glucuronidase, clotting factor IX, and erythropoietin were transferred to muscle using myoblasts.

Lysosomal storage diseases are a subset of disorders that may be appropriate for muscle-mediated gene therapy (80). These recessive disorders are caused by detrimental buildup of lysosomal enzyme substrates within affected tissues due to a single missing or dysfunctional lysosomal enzyme. Because these enzymes are marked with a specific targeting signal (mannose 6-phosphate) (81), missing lysosomal enzymes manufactured by muscle and delivered to the serum can be internalized by distant tissues and appropriately transported to lysosomes via mannose 6-phosphate receptors. By implanting into muscle genetically engineered primary myoblasts encoding β-glucuronidase, a lysosomal enzyme, 1 group was able to demonstrate in vivo expression of the recombinant protein in adult β-glucuronidase-deficient mice (77). Production and secretion of the missing lysosomal enzyme by muscle led to correction of phenotypic abnormalities in the liver and spleen of treated animals.

A second disorder well suited to myoblast-mediated gene therapy is hemophilia B. Hemophilia B is a blood-clotting disease caused by a deficiency of a protein, clotting factor IX. Because conventional protein replacement therapies face drawbacks, including the necessity for frequently repeated

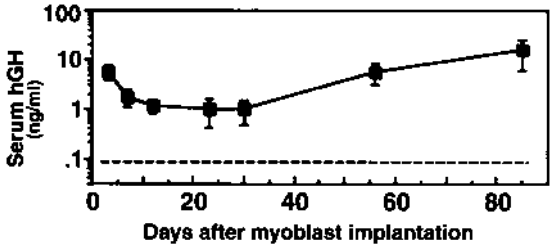

Figure 5 Systemic delivery of human growth hormone. A population of C2C12 myoblasts retrovirally transduced with hGH were implanted into hind limbs of 24 syngeneic mice, and serum hGH levels were monitored by radioimmunoassay of tail blood. Greater than 90% of the implanted cells expressed and secreted hGH as determined by clonal analysis in culture. Each point represents the mean ± SD for 4 to 24 mice; the dashed line shows the mean ± SD for serum samples taken from five uninjected control mice. Expression of hGH by implanted myoblasts persisted for at least 85 days in vivo. (Reprinted with permission from Ref. 53. Copyright © 1991 American Association for the Advancement of Science.)

treatments and the risk of contaminating blood-borne pathogens in plasma-derived factors, gene therapy may provide a safer and more convenient alternative (82). Initial studies in which C2C12 myoblasts were transduced with a gene encoding human factor IX and implanted into immunocompetent mice led to a peak expression of recombinant protein (1 μg/mL) at day 12, and subsequent decline back to basal levels thereafter (55). The drop in human factor IX expression was shown to be due to production of specific antibodies targeted against the protein in wild-type mice. Other experiments (56–58) demonstrated that primary myoblasts engineered to constitutively express factor IX led to stable, low-level production of the protein in immunodeficient nude or SCID mice for many months. Another study (51) achieved stable production of human factor IX at therapeutic levels in SCID mice, using a promoter with muscle creatine enhancers to drive high levels of muscle-specific expression, for at least 8 months. Of importance, recombinant factor IX manufactured in muscle undergoes the gamma carboxylation required for functional activity of the protein (55,56). This finding demonstrates that muscle cells have efficient mechanisms for posttranslational modifications normally carried out by other tissue types such as liver. Moreover, the problems with immunogenicity are likely to affect only a percentage of hemophiliacs, as not all are null mutations but have some, albeit reduced, level of factor IX (83). Until recently, only dog models were available, however, now a mouse model that lacks factor IX has been created by homologous recombination (84), which should facilitate future preclinical gene therapy studies.

A third class of disorders for which myoblast-mediated expression of recombinant proteins into the circulation may be beneficial is in the treatment of erythropoietin (Epo)-responsive anemias. Recombinant Epo replacement therapy has been employed for successful treatment of anemia associated with end-stage renal disease (85) and is being tested as a therapy for a broad array of other anemias (86). Epo is a mammalian hormone that controls the production of erythrocytes, hemoglobin-carrying cells that deliver oxygen to tissues of the body (87). Anemic patients can currently be treated by repeated administration of recombinant Epo; such treatments, however, require frequent hospital visits by patients and are costly. Thus, Epo delivery by gene therapy could provide patients with long-term delivery of the protein, eliminating the need for multiple treatments. However, as with many gene therapies, regulated expression is desirable for Epo, because dosage must be tailored to the particular application and to the individual patient.

Studies of muscle-mediated delivery of Epo by gene therapy appear promising. Epo-secreting primary or C2 myoblasts have been introduced bilaterally into skeletal muscles of mice (77,78). Implantation of engineered cells led to an elevated hematocrit for 3 months, a direct measure of Epo production. At 3 months posttransplantation, implanted myoblasts were observed to have fused and fully differentiated into myofibers (78). Moreover, in an animal model of renal failure in which anemia is induced by nephrectomy of immunocompromised nude mice, injection into muscle of C2 myoblasts secreting human Epo led to reversal of the anemic phenotype (79). Levels of recombinant serum Epo measured by enzyme-linked immunosorbent assay (ELISA) remained elevated for the 2 months during which the animals were assessed following myoblast implantation. These studies lend credibility to using myoblast-mediated expression of recombinant Epo as a viable treatment for anemias.

Thus, myoblast-mediated gene transfer appears to be well suited for expression of recombinant proteins to the circulation. Unlike applications aimed at treating inherited myopathies, not all fibers need to be transduced with the gene of interest to achieve a therapeutic effect. Indeed, such therapies can be highly localized to a particular region of a single muscle. For a variety of disorders where patients may benefit from delivery of a recombinant gene product to the bloodstream, myoblast-mediated gene transfer to muscle tissue appears to be a promising treatment method. Stable, long-term expression of physiological levels can be achieved with therapeutic effects, and because there is no immune response, repeated administration of genetically engineered myoblasts is possible, unlike AAV or adenoviral gene delivery.

C. Vascular Insufficiencies and Cancer

Since the 1990s, a great deal has been learned about growth factors that induce angiogenesis, the sprouting of new blood vessels from preexisting vessels. There has been much interest in the use of angiogenic factors to stimulate new vessels to grow as a treatment for maladies including stroke, peripheral arterial disease, and myocardial infarction. As the genes that encode these proteins have been cloned, the concept of thera-

peutic angiogenesis has moved quickly into the realm of gene therapy and clinical trials are already underway as low levels provide therapeutic effects. Factors produced by genetically engineered myoblasts are continuously produced by contrast with injection of pure proteins, naked DNA, and viral vectors, and may be advantageous.

The angiogenic factor that has received the most attention to date is vascular endothelial growth factor (VEGF), a potent mitogen that was isolated by virtue of its ability to stimulate growth of endothelial cells and to increase permeability in vascular endothelium (hence its other designation, vascular permeability factor) (88–92). VEGF plays an important role in the induction of angiogenesis by tumors (93), and in the angiogenic response of normal tissue to decreased oxygen availability. VEGF is also known to serve as a critical signal during the initial embryonic development of the vasculature by a process known as vasculogenesis, or the *de novo* growth of blood vessels from precursor cells (94,95). In this case, VEGF induces endothelial cell migration via specific receptors. Therefore, VEGF is a crucial regulator of both modes of growth and development of the vasculature pre- and postnatally.

Because of the potential clinical benefits of stimulating new blood vessel growth, much effort in recent years has been invested in the delivery of VEGF to tissues that are insufficiently vascularized. Injection of VEGF protein has resulted in angiogenic sprouting of vessels in muscle that was partially deprived of blood and oxygen, and therefore ischemic (96–98). However, presumably because of vascular permeabilizing and/or vasodilating properties, bolus injections of the protein have been reported to be deleterious, causing hypotension (99,100). As a result, recent investigations have assessed the feasibility of localized delivery of VEGF by gene transfer using plasmid DNA injection or adenoviral vectors. Both of these delivery methods lead to transient production of the recombinant protein, and to angiogenic sprouting from preexisting vessels in matrigel in vitro (101,102), in adipose tissues in vivo (103), as well as in ischemic skeletal or cardiac muscle (104–107). These results have led to clinical trials of VEGF gene delivery for ischemic heart and limb diseases (104,108,109).

The effects of long-term stable production of VEGF were recently investigated using the myoblast-mediated gene transfer techniques described above (110). This resulted in many unexpected findings. Myoblasts were transduced with a retrovirus carrying a murine cDNA encoding the heparin-binding $VEGF_{164}$ and injected into the muscles of SCID mice. A physiological response to VEGF was observed in every mouse that received VEGF-producing cells. At day 11 postimplantation, mice appeared outwardly normal and no differences were observed between VEGF and control muscle upon dissection. However, histological analysis of frozen muscle sections revealed that the implantation sites of VEGF-expressing myoblasts, but not control myoblasts, were invariably associated with regions of infiltrating mononuclear cells, identified by fluorescent antibody staining to multiple markers as endothelial cells and macrophages. By days 44–47, 100% of legs injected with VEGF myoblasts contained large hemangiomas composed of vascular channels and pools of blood, whereas control legs appeared normal (Fig. 6). These results have been reproduced in the heart following myoblast implantation into myocardium (111). However, implantation into muscle of clonal populations of myoblasts that express lower levels of VEGF results in an increased number of vessels without associated hemangioma formation (C. Ozawa et al., submitted). Interestingly, if the implanted cells are a mixture of myoblast clones expressing different levels of VEGF, due to genomic differences in retroviral insertion, the induced vasculature ranges from seemingly normal capillaries to hemangioma-like vascular sacs. This reveals a surprisingly localized response to VEGF on a microscopic scale and shows the importance of not exceeding threshold levels of VEGF, not only on a tissuewide level, but also on a per cell level. These studies demonstrate that myoblast-mediated VEGF gene delivery is extremely potent and provide evidence that this single growth factor can lead to a cascade of events resulting in the formation of complex tissues of multiple cell types. These results also show for the first time that exogenous VEGF expression at high levels or long duration can have deleterious effects, a factor of importance as clinical trials of VEGF gene delivery by plasmid DNA injection or adenoviral-mediated delivery are underway. Moreover, because myoblast implantation affords higher expression levels of longer duration than other gene transfer techniques, a physiological response to VEGF was observed in nonischemic muscle for the first time. Thus, the dose and duration of VEGF expression appear critical in determining a range of effects.

These results point both to the potency of myoblast-mediated gene transfer, and the necessity of regulation of recombinant gene expression for gene therapy applications. In the case of myoblast-mediated delivery of VEGF, too much of a good thing clearly can lead to adverse and unwanted effects. Gene therapy has usually been plagued by insufficient levels of the protein of interest. However, these VEGF results illustrate that current methods of gene delivery can be limited by a lack of ability to control gene expression. Both the ability to increase expression levels if an insufficient amount of a recombinant protein is being produced, and the option to intentionally reduce or cease expression, are likely to be necessary for the health of the patient in many cases.

In addition to studies aimed at triggering the growth of new blood vessels, other experiments are currently geared toward preventing blood vessel development in special circumstances. Because tumor growth and metastasis require persistent new blood vessel growth (112,113), therapies targeted at blocking this growth could lead to an arrest of tumor development. One of the most promising avenues for preventing angiogenesis in tumors may lie in the utilization of recently discovered antiangiogenic agents.

Early in the 20th century, it was first noticed that primary tumors are able to suppress the growth of a second tumor inoculum (114). Resistance to secondary tumor challenge was shown to be inversely proportional to the size of the second tumor inoculum, and directly proportional to the size of the

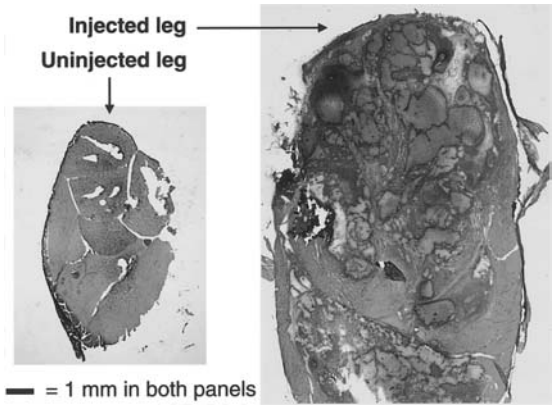

Figure 6 Formation of vascular structures in VEGF-myoblast-implanted legs. Myoblasts expressing the murine $VEGF_{164}$ gene were injected into mouse hindlimb. Histological analysis of injected muscles were conducted at day 44–47 postimplantation using hematoxylin/eosin staining of cryostat sections. Uninjected control legs were normal both in size and in morphology (left panel), whereas legs injected with VEGF myoblasts (right panel) were greater than twice the diameter of control legs, and consisted primarily of hemangioma and pools of blood. Both panels are shown at the same magnification. These results demonstrate the importance of regulating recombinant gene expression in gene therapy applications. (Adapted and reprinted from Ref. 110 with permission, copyright 1998 Cell Press.)

primary tumor (115,116). Moreover, removal of certain tumors can lead to rapid growth of metastases (117). Isolation of fractions taken from serum and urine that were capable of inhibiting endothelial cell proliferation in vitro and metastatic tumor growth in vivo led to the discovery of two antiangiogenic proteins, angiostatin (118) and endostatin (119), proteolytic products of plasminogen and collagen XVIII, respectively. A recent study demonstrated that viral vectors encoding angiostatin cDNA could inhibit endothelial cell proliferation in vitro and glioblastoma growth in vivo (120). An interesting therapy for cancer could be to engineer myoblasts to express these proteins, such that their secretion may inhibit growth of tumors at distant sites. Reconfirmation that desired blood vessel synthesis at sites of injury, for example, is not impaired would be critical. Because angiostatin and endostatin are difficult to produce in adequate amounts in bacteria, gene therapy protocols will be invaluable for discerning their biological function and possible application as anticancer agents in vivo.

V. REGULATABLE RETROVIRAL VECTORS AND THE RETROTET-ART SYSTEM

Until recently, all vectors employed in gene therapy protocols have depended on constitutive promoters to drive expression of the transgene. As made abundantly clear in the recent study of the effects of myoblast-mediated delivery of VEGF on normal adult muscle (110), regulation of gene expression is extremely important for safe treatment of patients. Both delivery by plasmid transfection or adenoviral vectors, the 2 VEGF gene transfer techniques currently being used in clinical trials, typically lead to transient gene expression that may be desirable in the case of angiogenic gene delivery. However, rather than count on inherent but uncontrollable limitations of the gene delivery systems, it may prove better to use a system that delivers sustained and excessive levels, but has been modified with regulatable control elements. This theoretically allows levels and timing of expression to be tailored to those that are deemed optimal on a case-by-case basis. An advantage of retrovirally transduced myoblast implantation is that it allows localized delivery of a recombinant gene at sustained levels; addition of inducible elements to retroviral vectors provides a mechanism for fine-tuning gene expression to the physiological levels required.

There are 4 characteristics that an ''ideal inducible system'' should possess. First, the regulatable system should demonstrate specificity—it should not require endogenous factors for activation, or interfere with cellular regulatory pathways. Second, the system should be efficient—demonstrating induction to high levels of gene expression from starting low basal levels, and the potential for repression back to uninduced levels. Third, it must be dose dependent, responding to its inducer by modulating its expression in a sensitive and homogeneous manner. Last, none of its components should elicit a host immune response or be toxic. To date, there are 4 regulatable systems displaying some or most of these characteristics: the ecdysone, RU486, FK506/rapamycin, and tetracycline-inducible systems. The first 3 systems are derived from members of the nuclear receptor superfamily (for ecdysone and RU486) or immunosuppressant compounds (for FK506/rapamycin) (for review, see (121,122)), and they may have potential effects on host genes in some cases. This section focuses on the attributes and recent advances of the tetracycline system, which has been extensively studied in our laboratory and has already been incorporated into myoblast implantation strategies.

The tetracycline-inducible system was originally developed by Bujard and coworkers (123,124) and has become one of the most widely used methods of regulating gene expression to date. All elements of the system are prokaryotic; thus, pleiotropic effects and endogenous ligands are avoided. In addition, because the inducer becomes an integral part of the transactivator directly responsible for turning on gene expression, there are no intermediate steps in the induction pathway. The tetracycline system thus allows for a more direct correlation between the amount of transcription factor capable of binding DNA, and the concentration of exogenous inducer [tetracycline (tet) or its synthetic analog doxycycline (dox)]. The pharmacokinetics of tet are well understood, and at the levels required for the inducible system, are well known to be safe for human use.

In its original and simplest form, the tet transactivator (tTA) is a hybrid factor comprising a bacterial tetracycline repressor (tetR) and the viral transactivator domain VP16 (123). When bound to tet, tTA is prevented from binding to tet operator sequences juxtaposed to a minimal promoter, and gene expression is turned off. In the absence of tet, tTA is free to bind to the inducible promoter, and gene expression is induced up to 5 or 6 orders of magnitude. A relatively recent modification of the system allows for induction of gene expression in the presence, rather than absence, of tet (124). A second chimeric protein containing a mutated version of tetR was developed and designated as ''reverse'' tTA (rtTA); this transactivator binds to tet operator sequences in the presence of tet. Both tTA and rtTA have been demonstrated to efficiently regulate expression in tissue culture, fruit flies, and mice (125–128).

A major advance in broadening the utility of the tet system was the employment of retroviruses. Retroviral gene delivery is much more rapid and efficient than transfection-using plasmids. Retroviral vectors also do not form concatemers and thus should not form a repressive chromatin environment sometimes associated with plasmids (129). Genes can be introduced into tens of thousands of myoblasts at high efficiency, generating polyclonal populations within a week (50), an advantage over the few stable clones routinely obtained. For these reasons, retroviruses are well suited for delivery of tet-inducible systems to primary cells isolated directly from tissue. Initial studies using tet-regulatable cassettes, however, met with numerous problems. In one case, the inclusion of an autoregulatory feedback loop necessitated high background levels of expression in order to ''jumpstart'' the system (130). In other cases, overcomplexity of transcription and translation units produced low viral titers (131–135).

Bohl and colleagues (136) first overcame this problem by using simplified retroviral vectors in which the necessary elements were dispersed over more than 1 retroviral vector. In this study, 1 retrovirus encoded rtTA, whereas the other contained an inducible Epo cassette. After multiple rounds of infection, primary myoblasts exhibited induction of about 200-fold in expression of the protein. When the engineered myoblasts were transplanted into mice, Epo expression could be repetitively turned on and off over a 5-month period by controlling levels of dox in drinking water. In an improvement of this approach, inclusion of a selectable marker such as green fluorescent protein (GFP) allows for purification by flow cytometry of regulatable populations of cells (128) (Fig. 7).

Recently, 2 additional advancements of the tet system have increased its applicability for gene therapy purposes. The tetR transcriptional elements are modular; one may replace the VP16 transactivator domain of tTA, for instance, with a KRAB transrepressor domain to create a tet-regulated repressor of transcription (137). Expression of 2 tet modulators within the same cell, however, leads to formation of nonfunctional heterodimers because the modulators have identical dimerization domains (Fig. 8) (138). Based on sequence information and known crystal structures of tetR as well as mutational analysis (139–141), mutually distinct dimerization domains deriving from separate classes of Gram negative bacteria have been identified (142,143). The ability to engineer tet modulators with specific dimerization domains allows tet activators and tet repressors to be expressed within the same cell without risk of forming a nonfunctional heterodimer. The development of such a tetracycline-inducible retroviral system, designated the RetroTet-ART (activators and *r*epressors expressed *t*ogether) system (142), allows for gene expression to be completely extinguished or induced in a fully dose-dependent manner—as a result, the dynamic range of gene expression has been greatly increased (Fig. 9). This improvement is a significant advantage in applications where basal expression from the inducible promoter must be extinct. The RetroTet-ART system was demonstrated to be able to reversibly silence expression of p16, a growth arrest protein (142).

In a second modification of the tet-inducible system, the DNA-binding domain of tetR was altered to interact with a modified tet operator sequence (143). The original and adapted binding sequences were engineered into tTA and rtTA proteins harboring distinct dimerization domains. By placing 2 separate genes under control of old and new tet operator sequences and expressing both of the modified tTA and rtTA proteins, Baron and colleagues were able to either repress expression of both genes or express either gene alone simply by changing the dox concentration (Fig. 10) (143). A means of turning on both genes at once has yet to be achieved. Thus, the activity of two different genes can be reversibly controlled in a mutually exclusive manner.

In summary, tet-regulatable retroviral systems are capable of being repressed and expressed in a fully inducible manner both in vitro and in vivo. Tet has been used for decades in humans and animals, and only at higher doses above those required for induction of transgenes have few if any deleterious effects been observed. In addition, when the rtTA protein was delivered to mice by ex vivo gene delivery using myoblasts, no immune response to foreign elements was observed (136). Retroviruses are efficient means of delivering tet-regulatable vectors to large numbers of primary cultures of cells, including myoblasts. Thus, there is much reason to believe that the tet-regulatable retroviral systems, in conjunction with myoblast-mediated gene delivery, may be well suited for gene therapy applications in humans in the future.

VI. FUTURE PROSPECTS FOR MYOBLAST-MEDIATED THERAPIES

A. Inherited Myopathies

As noted earlier, a problem in using the ex vivo delivery approach to treat inherited myopathies is that for such therapies to be effective, a large proportion of skeletal muscle must be targeted. Direct intramuscular implantation of myoblasts leads to fusion of the injected myoblasts to a majority of fibers in the region of the injection site; the number of fibers to which genes are delivered by this approach decreases with increasing distance from the site (70). Thus, for a sufficiently large percentage of fibers to be treated, many closely spaced injections

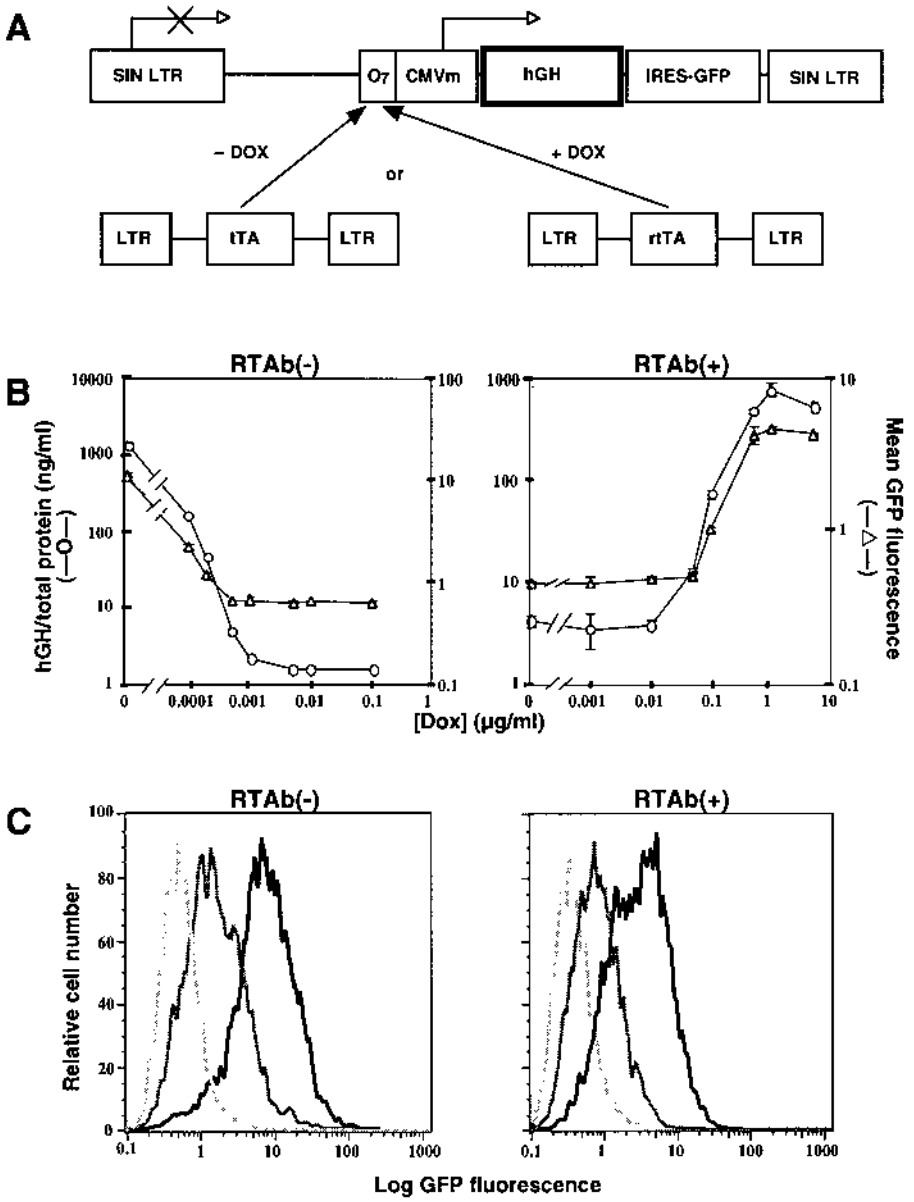

Figure 7 Tetracycline-inducible expression using tTA and rtTA. (A) Schematic of a binary retroviral system allowing tetracycline-inducible expression of both human growth hormone (hGH) and green fluorescent protein (GFP). The expression of both proteins is ensured by the use of an internal ribosomal entry site (IRES), allowing both genes to be encoded within the same mRNA transcript. The reporter virus contains a self-inactivating (SIN) retroviral backbone to avoid interference of the viral long terminal repeat (LTR) with the tet-responsive promoter (O_7-CMVm). The diagram represents the system after integration into the chromosome; hence, the SIN LTR exists in both the 5′ and 3′ positions. Tet-sensitive transactivators (either tTA or rtTA) are provided constitutively from a second retrovirus. (B) Dose response of the binary tet-inducible system shown in (A). RTAb(−) cells transduced with tet reporter virus and tTA virus, and RTAb(+) cells transduced with tet reporter virus and rtTA virus, were assessed for their dox dose response of hGH (○) expression and GFP (Δ) expression. Both systems exhibit concentration dependence over several orders of magnitude. (C) Histogram plots of GFP expression obtained from FACS analysis are shown in overlay at three selected doses [RTAb(−): 0.1 μg/mL (light gray), 0.001 μg/mL (dark gray), and 0 μg/mL (black); RTAb(+): 0 μg/mL (light gray), 0.1 μg/mL (dark gray), and 5 μg/mL (black)]. These plots show that with changing concentrations of dox, populations uniformly shift to intermediate and high levels of expression. (Adapted and reprinted from Ref. 128 with permission of the Proceedings of the National Academy of Sciences USA, 2101 Constitution Ave., NW, Washington, DC 20418. Reproduced by permission of the publisher via Copyright Clearance Center, Inc.)

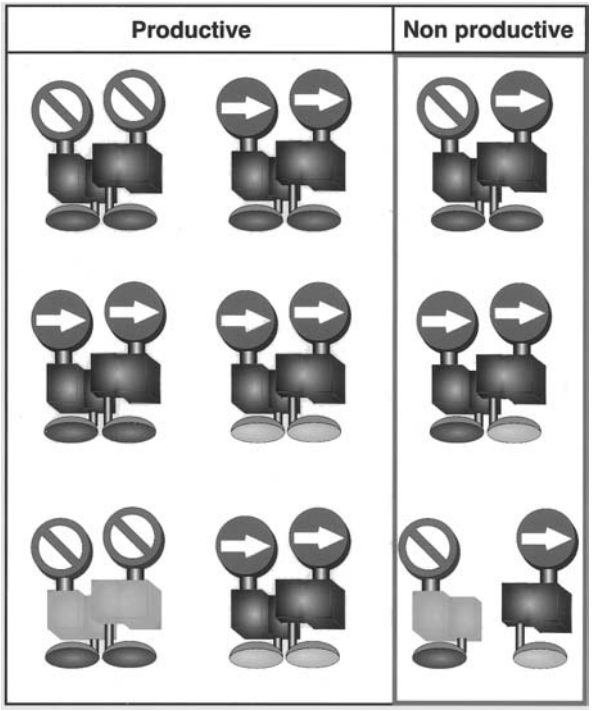

Figure 8 The need for tet modulators with distinct dimerization domains. Coexpression of tetR fusion proteins with different functional domains such as repressor domains (represented in the top row by the ''do not enter'' sign), and activator domains (represented by the ''go'' sign), or DNA-binding domains with distinct specificity (symbolized in the middle row by the light gray and dark gray ''feet''), leads to formation of both functional homodimers and nonfunctional heterodimers. Such nonfunctional heterodimers can be eliminated by engineering distinct dimerization domains into the tetR portion of the tet modulators (symbolized by the dark gray and light gray shaded midsections in the bottom row). (Reprinted from Ref. 138 with permission of the Proceedings of the National Academy of Sciences USA, 2101 Constitution Ave., NW, Washington, DC 20418. Reproduced by permission of the publisher via Copyright Clearance Center, Inc.)

would be necessitated. This requirement imposes a major limitation to the utility of myoblast-mediated gene transfer in treating human muscular dystrophies, which often affect cardiac and diaphragm muscles as well as skeletal muscles. Unless a myoblast population is isolated that can efficiently migrate to damaged or degenerated muscle, this approach seems too inefficient to be useful.

Recent observations that cells originating from bone marrow can become incorporated into regions of induced muscle regeneration has been met with much interest (144–146). This finding was significant because it elucidated the possibility that such bone marrow-derived cells can travel through the circulation and enter into skeletal muscle tissue (147). A possible solution to the problem of targeting skeletal muscles throughout the body may lie in introducing genetically engineered muscle precursor cells to the circulation, where they can reach muscles throughout the entire body. One study has examined the feasibility of intra-arterial delivery of genetically labeled, immortalized L6 myoblasts to skeletal muscle (71). After infusion of these cells into the arterial circulation, a small number of labeled fibers were observed in skeletal leg muscle, showing that the circulation may be capable of delivering muscle precursor cells to differentiated myofibers, although some were also found in the lung. Alternatively, if muscle stem cells of the bone marrow could be isolated, genetically engineered ex vivo and injected back into the patient, they could serve as a continual pool of circulating therapeutic effectors for the treatment of myopathies.

The existence of a muscle stem cell has been suggested from several pieces of evidence. Populations of cells that are capable of self-renewal and that give rise to differentiated cells have been identified both in the myogenic C2 cell line (148), and in clones of human myoblasts (149). In addition, a recent paper showed, using 2 genetic markers with different modes of inheritance to examine the fate of myoblasts transplanted into skeletal muscle, that only a discrete minority of transplanted myoblasts participate in regeneration of host muscle (150). This minority population of cells appears to divide slowly in vitro, but proliferates rapidly in vivo upon transplantation into regenerating muscle (150). If methods for characterizing and isolating this muscle stem cell population could be devised, such cells could be genetically engineered ex vivo and then introduced to patients, either by infusion into the circulation or through introduction to the bone marrow.

B. Circulating Therapeutic Proteins

The utility of myoblast-mediated gene delivery has broadened to include disorders that benefit from long-term secretion of recombinant proteins into the circulation, including treatment of lysosomal storage deficiencies, hemophilia B, anemias, and possibly cancer. For application of myoblasts in delivering genes encoding recombinant secreted proteins, a hurdle limiting its utility in the therapeutic realm is the necessity of using syngeneic cells to avoid immunological rejection of transplanted cells (151). Although myoblasts may be both isolated from and implanted back into the same individual, such procedures are both time consuming and costly. An alternative strategy would be to encapsulate myoblasts in an immunoisolated environment prior to implantation. Using this approach, myoblasts are enclosed within a matrix, for example, an alginate matrix (although other materials may be used), that allows secreted proteins to leave the capsules. The recipient's immune cells are prevented from coming into contact with the myoblasts, obviating the need for a genetically identical donor. This technology has been shown to be effective in delivering myoblasts engineered to secrete mouse growth hormone (27) and human factor IX (28) intraperitoneally. Encapsulated myoblasts were shown to be retrievable as long as 213

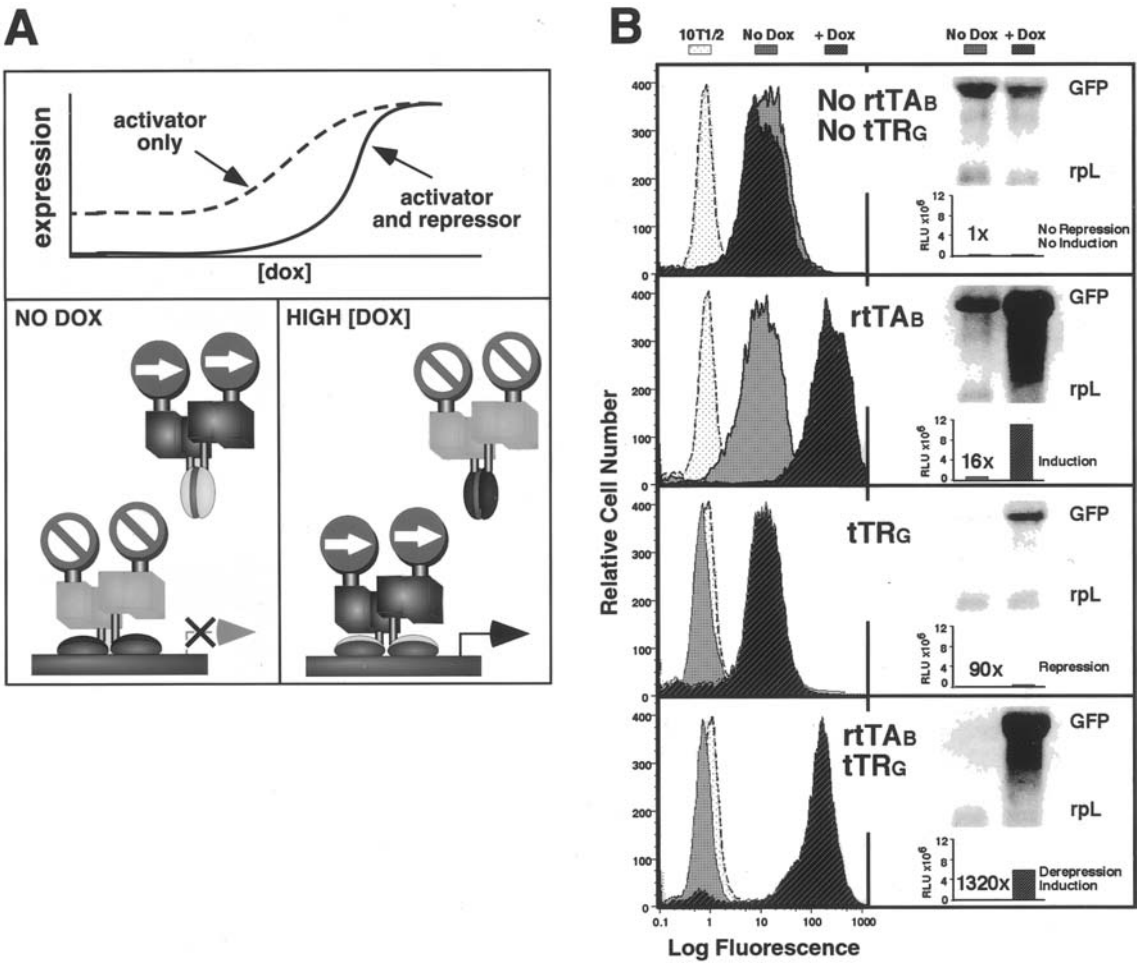

Figure 9 The RetroTet-ART system. (A) By coexpressing in the same cells a repressor and an activator that respond oppositely to dox and that do not heterodimerize because of different dimerization domains, the basal expression level of genes under tet control can be reduced without affecting the fully induced level. The net result is an increase in the dynamic range of the tet system (Reprinted from Ref. 138 with permission of the Proceedings of the National Academy of Sciences USA, 2101 Constitution Ave., NW, Washington, DC 20418. Reproduced by permission of the publisher via Copyright Clearance Center, Inc.). (B) Proof of concept of the RetroTet-ART system is demonstrated by FACS (left) and Northern blot (right) analysis. 10T1/2 fibroblasts were transduced with the GFP reporter retrovirus. Subsequently, cells were transduced with the transactivator retrovirus (b), the transrepressor virus (c), or with both transactivator and transrepressor virus (d). With the addition of both transactivator and transrepressor, the dynamic range of gene expression is increased. Gene expression can be fully extinguished, and induced to maximal levels, as shown in (d). (From Ref. 142 with permission)

days postimplantation. These cells were found to be fully viable and capable of secreting recombinant proteins ex vivo at undiminished rates even at this late time point (28). More recently, encapsulated primary myoblasts were used to deliver VEGF to mice subcutaneously and intraperitoneally, causing an angiogenic response (29). This type of technology provides a promising method of attaining nonautologous gene therapy, in which universal donor cells can be created simply by encapsulation in a benign, immunoprotective environment.

VII. SUMMARY

The types of disorders considered as potential targets for gene therapy has changed with the development of the field. Initially, most studies centered on developing therapies for single gene defect disorders; however, these present the greatest challenges. Thus, increasingly more attention is being paid to complex diseases involving more than 1 gene, such as cardiovascular disease and cancer, because these appear more tractable. In addition, although efforts attempting to correct genes

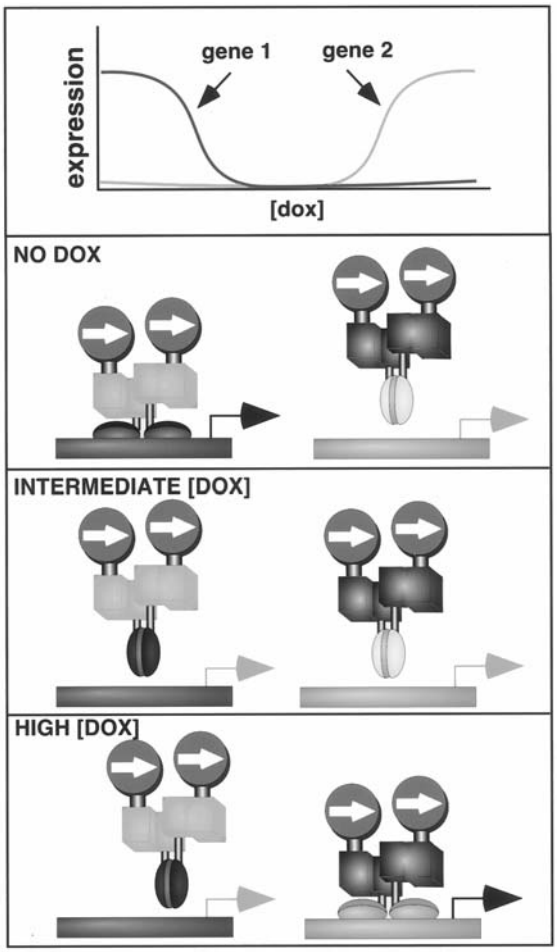

Figure 10 Tet-regulated expression of two separate genes. By coexpressing two tetR-based activators that contain DNA-binding domains with distinct specificity, respond oppositely to dox, and do not heterodimerize, two independent genes can be regulated by the same inducer. Because of the characteristic dose response of the wild-type and ''reverse'' tetR, the expression of each gene can be turned off at an intermediate concentration of dox and activated at markedly different dox concentrations. (Reprinted from Ref. 138 with permission of the Proceedings of the National Academy of Sciences USA, 2101 Constitution Ave., NW, Washington, DC 20418. Reproduced by permission of the publisher via Copyright Clearance Center, Inc.)

that are defective are under development, currently methods for complementing their defects through recombinant expression of related genes are more readily achieved. As the strategies for gene therapy develop in complexity, the methods available for treatment of disorders must also increase in their level of sophistication. Because of its many advantages, myoblast-mediated gene delivery may be a method well suited for addressing these needs for certain types of diseases. Myoblasts can be multiply transduced ex vivo with a variety of retroviral cassettes, each containing separate genes. The availability of improved tet-inducible retroviral vectors allows for fine control of recombinant gene expression levels. The 2 systems together—ex vivo gene transfer using myoblasts and regulatable retroviral vectors for transducing myoblasts—contribute a powerful toolbox with which to develop gene therapies for a number of human diseases. If myogenic stem cells prove to be readily isolated, cultivated, and able to migrate to muscle tissue, a large percentage of muscle could be targeted with relative ease, allowing for broad application in the treatment of both inherited myopathies and nonmuscle-related disorders.

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9

Vaccinia Viral Vectors

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I. INTRODUCTION

Vaccinia virus has been used clinically as a vaccine for smallpox since the late 18th century, and thus is associated with a rich history and extensive clinical experience (1,2). In 1798, Edward Jenner demonstrated protection from smallpox by vaccination with the cowpox virus obtained from infected milk maids. In the early 19th century, this evolved into the use of vaccinia virus (3). The precise origin of vaccinia virus is difficult to identify because the virus has no known natural host, leading some researchers to suggest that it may have arisen from mutations in the cowpox or smallpox viruses (4). However, it is more likely that vaccinia represents a distinct strain that is extinct in its natural host or so rare that it is difficult to identify naturally (5).

Vaccinia virus was widely used as a vaccine for the eradication of smallpox until 1978. Since then, it has been used experimentally as an in vitro gene expression vector and to express foreign genes as a vaccine for infectious agents and cancer (6). Vaccinia has many advantages over other viruses as a vector for gene delivery. Vaccinia has a wide host range. Its genome has been completely sequenced, facilitating the creation of recombinant vectors. It can hold up to 25 kb of foreign DNA without a need for viral deletions (7). Recombinant vectors are easily produced in high titers for use in vivo (8).

Vaccinia virus has also been modified to carry various antigens, cytokines, and immunostimulatory molecules (9). In fact, it has become evident that several properties make it useful as an oncolytic virus for cancer gene therapy (10). Any cell infected with the virus is rapidly killed, and cell-to-cell spread is efficient. Also, a natural tumor tropism exists in animal models. Applications requiring long-term gene expression, however, are not feasible with vaccinia. This chapter reviews the relevant aspects of vaccinia biology necessary for its use as a vector, and reviews the preclinical and clinical development of vaccinia virus as a vector for gene therapy.

II. BIOLOGY OF VACCINIA

Poxviruses are classified into 2 subfamilies, chordopoxvirinae (vertebrate poxviruses) and entomopoxvirinae (insect pox viruses), and at least 46 species (11). The classification scheme is based on host range, sequence homology, and antigenicity. Vaccinia virus is a member of the orthopoxvirus genus. It is genetically distinct from both cowpox virus and variola virus (smallpox). All members of the orthopoxvirus genus have immune cross-reactivity and are genetically stable. This allowed for the complete eradication of variola virus in 1977 (last case of endemic smallpox).

Multiple strains of vaccinia viruses exist (Table 1). As vaccination became widespread throughout the world, numerous centers produced and maintained the vaccine in different ways, resulting in numerous strains, which differ in characteristics, pathogenicity, and host range. The New York City Board of Health strain was obtained from England in 1856 and was originally used for smallpox vaccination in the United States (5). The Western Reserve (WR) strain is a laboratory derivative of this strain and appears to be one of the more virulent strains in laboratory animals and nonhuman primates.

It has not been used in patients to date. Another derivative, the Wyeth strain, was produced by Wyeth as a smallpox vaccine and is the backbone commonly used for experimental vaccines in clinical trials. The modified vaccinia ankara (MVA) strain was developed through multiple rounds of infection in avian cells. This strain is highly attenuated and does not replicate in human cells (12).

As with all poxviruses, the vaccinia virus is a double-stranded DNA virus whose entire life cycle exists within the cytoplasm of eukaryotic cells. The virus contains an outer envelope, as well as an internal membrane, and it carries the enzymes required for initiation of transcription. The genome of the Copenhagen strain of vaccinia virus was completely sequenced and reported in 1990 (13); other strains have been sequenced subsequently (14). The genome consists of double-stranded DNA with inverted terminal repeats and a terminal hairpin loop, which mimics a large, circular, single-stranded DNA. The genome consists of 191,636 base pairs encoding approximately 2063 proteins of 65 or more amino acids in length. It is among the largest viruses in size, averaging 270 $\times$ 350 nm in the shape of a brick (15).

Vaccinia has two infectious forms: the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) (16). The infectious form responsible for cell-to-cell spread in vivo is the EEV. IMV is the form recovered during viral purification in vitro, as it is released on cellular disruption. Purification of EEV is quite difficult because the EEV envelope is too fragile to withstand the purification process. The process of cell entry by vaccinia is not well understood and is complicated by the fact that there are multiple forms of infectious virus, and multiple cell types and species infected. EEV and IMV bind different but unknown cellular receptors as evidenced by different binding efficiencies among cell lines, and the observations that the enzymatic digestion of cell surface molecules and the binding of specific monoclonal antibodies to cells affects binding of EEV and IMV differentially (17). Consistent with this, confocal microscopy has shown that IMV and EEV enter cells by different mechanisms (18). The IMV contains several proteins on its outer envelope, including A17L, A27L, and D8L (19–25). D8L was one of the first membrane proteins identified in the IMV. It is nonessential in the viral life cycle but may mediate IMV binding to cell surface chondroitin sulfate (23,24). A27L may mediate vaccinia interaction with cell surface heparan sulphate. Virus infection is inhibited by 60% in the presence of soluble heparin

Table 1 Vaccinia Strains Reported in the Literature

NYCBH	Tashkent	Ikeda
Wyeth	USSR	IHD
WR	Evans	DIs
Copenhagen	Praha	LC16
Lister	LIVP	EM63
MVA	Tian Tan	IC
		AS

(22). EEVs are believed to be responsible for cell-to-cell spread and long-range transmission of vaccinia virus in vivo (26). Six proteins (encoded by A33R, A34R, A36R, A56R, B5R, and F13L) are EEV specific (11). However, A56R has no effect on infectivity or spread if mutated (27).

It may be possible to circumvent normal receptor requirements by engineering vaccinia virus to bind to alternative cell surface molecules. Consistent with this, expression of an ScFv to erbB2 on the surface of the EEV (created as a fusion with A56R) was shown to bind erbB2 by enzyme-linked immunosorbent assay (27). Creation of a fusion protein between an ScFv and A56R is technically feasible and may direct binding of the EEV to a specific antigen or cell type. Fusions of other surface proteins, including B5R have been reported (28).

The life cycle of vaccinia is illustrated in Fig. 1. Vaccinia virus (as with all poxviruses) spends its entire life cycle in the cytoplasm of the host cell and has never been shown to integrate into the host genome. Vaccinia has very few interactions with host cellular proteins, allowing for rapid, efficient replication without negative effects from host cell defenses. The virus induces a profound cytopathic effect very soon after viral entry, as early viral enzymes completely shut down host cell functions. By 4 to 6 h after infection, there is almost complete inhibition of host protein synthesis. This allows for very efficient expression of viral genes and viral replication. In fact, approximately 10,000 copies of the viral genome are made within 12 h of infection (29). Half of these are incorporated into mature virions and released.

After vaccinia enters the cell, it undergoes a process of DNA uncoating and transcription begins. Three stages of transcription—early, intermediate, and late—have been described, each with its own specific promoters and transcription factors (30). The enzymes required for initiation of transcription are encapsulated in viral particles and released on viral entry. Proteins needed for viral replication are synthesized at the early (prereplicative) stage of infection. A DNA-dependent RNA polymerase is injected with the virus into the cytoplasm leading to the synthesis of early mRNA. Translation of this RNA forms early proteins, which are involved in uncoating of the viral DNA, DNA replication, and intermediate transactivation for transcription of intermediate mRNA. Intermediate mRNA is then expressed, which encodes for late transactivators leading to late mRNA synthesis. Late proteins include structural proteins for membrane formation and early transcription factors to be incorporated into the new virus particle. Only a relatively small number of proteins are required for DNA synthesis, making the system simple and largely autonomous.

Unlike many viruses, which rely on cellular machinery to replicate, vaccinia replicates virtually as an independent unit. Artificial transcriptional control for gene therapy applications is therefore challenging. It is clear, however, that some cellular protein interaction with viral transcription may occur. One group has identified a cellular protein transcription factor YY1, with vaccinia late promoter-binding activity (31). YY1 has been shown to bind the vaccinia ILl late promoter (32).

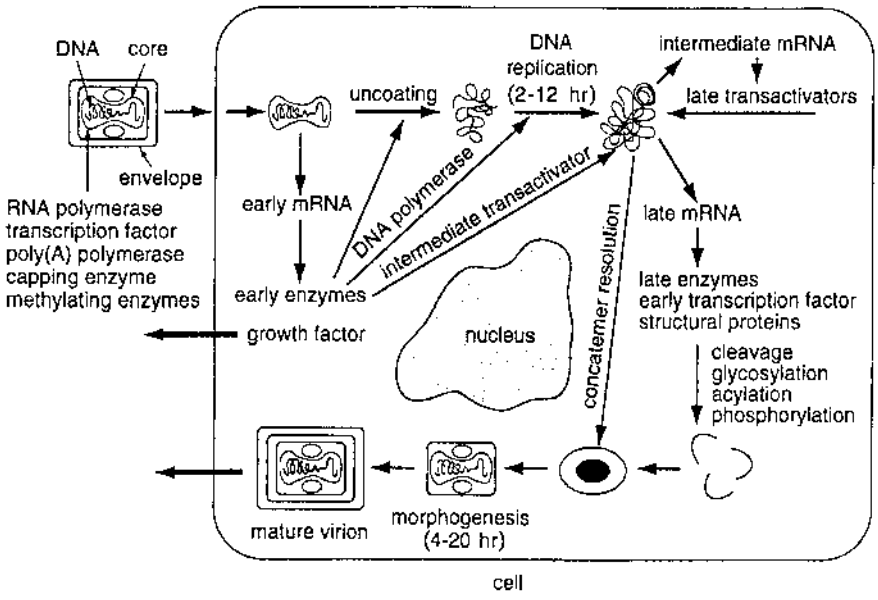

Figure 1 Schematic of vaccinia life cycle. (From Ref. 144.)

The vaccinia double-stranded DNA genome replicates in the cytoplasm, forming multiple concatamers of the genome. These concatamers are then resolved into individual genomes, which are encapsulated along with the early transcription factors into golgi-derived membranes. The first stage in the formation of infectious particles is the development of viral crescents composed of lipid and viral protein. To date, the origin of these crescents is disputed. Currently, the crescent is believed to be composed of a single lipid bilayer without continuity to cellular membranes (33). These crescents then coalesce into immature virus that lack infectivity. Immature virus then matures into IMV by condensation of the core and processing of core proteins (Fig. 2). IMV is transported to sites at which it becomes wrapped with 2 additional membranes. These membranes are derived from trans-golgi network membranes that have been modified by the inclusion of virus-encoded proteins and ultimately become part of the EEV outer envelope. These wrapped intracellular enveloped virus move to the cell surface where the outer membrane fuses with the plasma membrane, exposing the virus on the cell surface. If the virus is retained or reattaches it is called the cell-associated enveloped virus (CEV), but if released, becomes EEV. The A34R gene product plays a role in holding the virus to the cell surface. Mutations in A34R lead to increased EEV released and decreased CEV (34).

This entire life cycle occurs very rapidly. Initial RNA transcripts are detectible within 20 min of infection and DNA replication begins 1 to 2 h after infection. Within 12 h after infection, the majority of mRNA within the cytoplasm is from vaccinia-encoded genes. The entire replication cycle occurs in approximately 12 h (6).

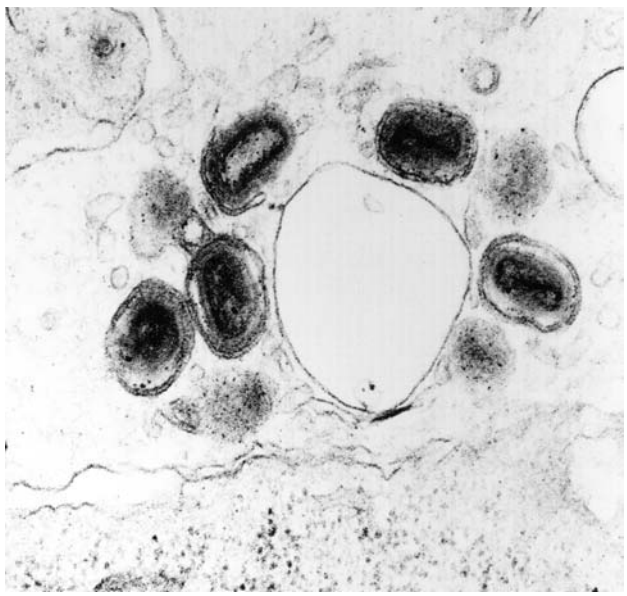

Figure 2 Electron micrograph of intracytoplasmic IMV form of vaccinia. The virions have a characteristic brick shape with a biconcave central core. (Courtesy of Maria Tsokos, MD, and Mones Abu-Asab, PhD, Laboratory of Pathology, NCI.)

III. CONSTRUCTION OF RECOMBINANT VECTORS

Homologous recombination occurs naturally during the replication of vaccinia virus, thus lending itself toward efficient insertion of foreign DNA. The creation of recombinant vaccinia vectors is relatively simple. The issues to be considered when creating a recombinant vaccinia vector include choosing a site for proposed recombination, a selection method(s), and a promoter for the foreign gene.

A shuttle plasmid is first created where a foreign gene expressed off a vaccinia promoter is flanked by vaccinia DNA sequences. Care must be taken that the foreign gene does not contain vaccinia transcription termination signals for early promoters (TTTTTNT) (35). The most common site of recombination has been the vaccinia thymidine kinase (TK) gene. Insertion of genes into the TK locus eliminates functional viral TK, leading to attenuation of the virus in vivo (36). Recombinations into numerous other loci have been performed, including intergenic segments such that no functional deletion occurs (37,38). The functional analysis of many vaccinia genes has been defined through insertional deletion.

A wide range of vaccinia promoters are available for expression of transgenes. It is necessary to use vaccinia promoters for creation of the recombinant vectors because these are specific for vaccinia polymerase. Eukaryotic promoters will not function in vaccinia infection because the host cell polymerase is not present in the cytoplasm where vaccinia transcription occurs. Several natural and synthetic early and late promoters have been described with various levels of activity (39–41). The native vaccinia promoters are generally very strong and compare favorably to other viral promoters used in other viral vectors. The synthetic early/late promoter described by Chakrabarti et al. (41) has led to consistent, reliable high levels of gene expression in numerous systems tested.

Several methods for selection of recombinant viruses are available. Growth in the presence of the thymidine analog BdUr can be used to select for a TK-negative phenotype in select cells after recombination into the TK locus (42). Others have commonly used the selection gene xanthine-guanine phosphoribosyltransferase, which allows for selective growth in media containing mycophenolic acid (43). Positive selection through replacement of an essential gene previously deleted from a backbone virus grown on permissive cell lines is also available (44). β-galactosidase and green fluorescent protein can aid in selection of recombinants. Once the shuttle plasmid is constructed, it can be transfected into a cell that has been infected with vaccinia. Homologous recombination leads to the insertion of the foreign gene into 0.1% of progeny virus genomes (6). The use of at least 3 rounds of selection ensures that there is no contaminating parental virus.

IV. IN VITRO GENE TRANSFER VECTOR

It has long been recognized that vaccinia is a valuable tool for expression of foreign genes in vitro (45,46). It is relatively easy to make recombinant viruses, and to grow and purify the virus. Large inserts can be accepted in the genome, and strong synthetic promoters lead to high levels of protein expression from infected cells. The virus has a broad tropism, and will infect and replicate in most mammalian cells. The high efficiency of expression with vaccinia obviated the need for other vectors, which permanently integrated into the genome and the cloning of high expressing cells. Infection with recombinant vaccinia leads to high levels of expression of foreign genes that are processed in the appropriate way such that their function can be studied. Vaccinia infection of cells at an multiplicity of infection (MOI) of 1.0 leads to >99% of cells expressing the gene of interest in most cell lines. Rather than make recombinants, simple plasmid transfection in a virally infected cell leads to efficient expression of foreign genes. The gene must be placed under control of a vaccinia promoter. The backdrop of vaccinia infection allows for cytoplasmic transcription, avoiding the inefficient process of trafficking into the nucleus. This leads to >90% of cells expressing foreign genes after simple plasmid transfection (47). This efficient transient expression system has been used for the functional analysis of innumerable foreign proteins over the years. Inactivation of the virus with psoralen and ultraviolet (UV) light can result in an efficient expression vector that does not cause a cytopathic effect (48). This may be important for the functional analysis of some proteins.

V. IN VIVO PATHOGENICITY AND BIODISTRIBUTION

Vaccinia virus has been used as a live vaccine in the smallpox eradication program, and more recently as a vaccine against cancer (49). It has not been widely accepted as a potential tumor-directed gene therapy vector, however, due to concerns regarding the safety of a systemically administered replicating virus. Although it is generally considered to be a relatively safe vector for vaccinations, a defined risk exists for generalized vaccinia, vaccinia-associated encephalitis, vaccinia necrosom, and eczema vaccinatum have been described in infants and the immunosuppressed population, specifically those with deficits in cellular immunity (50–55).

Vaccinia-associated encephalitis is a recognized complication of smallpox vaccination that can lead to death, and vaccinia can be recovered from the central nervous system (54). Classically, vaccinia infection in immunosuppressed patients leads to a progressive necrotic ulcer known as vaccinia necrosum. This ulcer can progress to destroy significant amounts of tissue, leaving exposed bone, requiring tissue grafts or amputation (56) (Fig. 3). Often this dramatic local infection does not lead to systemic viral spread. Patients with eczema, however, can get vaccinia infection of eczematous skin throughout the body (eczema vaccinatum). A large viral load such as this leads to fever and malaise and can lead to death from a ''septic syndrome.'' The exact cause of vaccinia pathogenicity and lethality in animal models is difficult to determine. Mice moribund from wild-type vaccinia infection have demonstrated high levels of circulating inflammatory cytokines, and it is

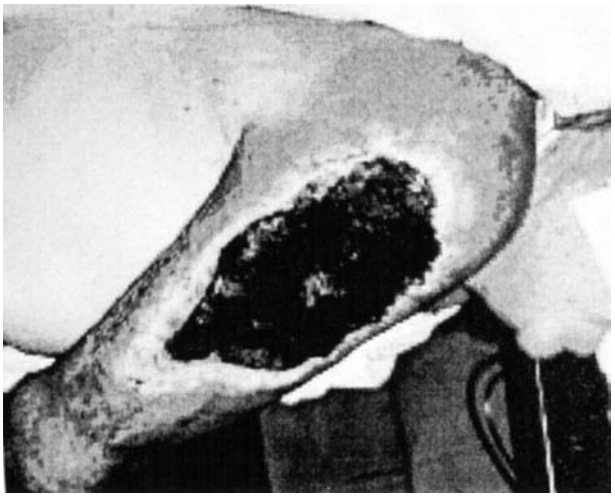

Figure 3 Example of vaccinia necrosum in a 66-year-old male 50 days after vaccination with a vaccinia melanoma cell lysate. The man had chronic lymphocytic leukemia. (From Ref. 145.)

suspected that the systemic inflammatory response syndrome plays a significant role in viral pathogenicity as opposed to specific organ dysfunction (Naiak et al., unpublished data, 1999).

The biodistribution of systemic injection of wild-type and TK-deleted vaccinia virus has been studied extensively in many animal models, including mice, rats, rabbits, and primates (10,57,58). The highest titers of recoverable virus are always from the ovary. Many fold less virus is recoverable from the liver, spleen, lung, and brain. Cutaneous pox lesions, which have high titers of vaccinia, can form. Mutations (e.g., TK deletion), which attenuate the virus by making it less efficient for replication in nondividing cells, are less recoverable from normal organs after systemic injection (59).

VI. HOST IMMUNE RESPONSE TO VACCINIA

The immune response to vaccinia viral vectors serves as our paradoxical friend and foe in attempting to develop them into effective vectors for gene therapy. On the one hand, the vigorous immune response is desirable because we believe that it enhances its potential as a vaccine. On the other hand, the vigorous immune response leads to premature clearance of the virus before adequate levels of replication have occurred, thus decreasing the level of transgene expression and possibly the overall efficacy.

Most viruses that infect human cells are also endemic in the population; therefore, many patients will have circulating antibodies against the viruses and preformed cellular precursors. Vaccinia is unique in that it is not endemic to humans, and because widescale smallpox immunizations terminated in the 1970s, young patients will not have been exposed to the virus. Most cancer patients, however, are older and have been exposed to vaccinia. As with other virus vectors, however, reinfection is possible after prior exposure. Laboratory workers and military personnel who undergo revaccination for smallpox usually form pox vesicles in the skin, despite prior vaccination. This has also been demonstrated in tumor vaccine trials in patients previously immunized (60). Workers at vaccine production plants in the past suffered from repeated skin infections with vaccinia and pox lesions in the skin. Some viruses can avoid circulating antibodies by mutating their coat proteins and changing serotype. This is not seen with vaccinia virus. However, vaccinia has evolved expression of immunosuppressive proteins (61). Viral surface proteins act as complement inhibitors, and the extracellular envelope is known to be almost completely resistant to antibody neutralization (62).

Both cellular immunity and neutralizing antibodies play a role in protection from vaccinia infection. The T cell response to vaccinia seems to be quite potent and is probably more important than antibodies in the primary host resistance to the virus. Progressive vaccinia correlates with a defect in cell-mediated immunity (63). In murine models, in the absence of a functional T cell population vaccinia is able to replicate and express genes within tumor cells at high levels for greater than 30 days (64). In an immunocompetent host, the window of gene expression only lasts for about 8 days with high levels of gene activity lasting approximately 4 days (58).

The success of vaccinia as a gene therapy vector relies on its efficiency in vivo. Vaccinia has developed a wide range of immune evasion strategies in order to survive in vivo (Table 2). Understanding and manipulating these factors may optimize the vector for clinical use. If one examines these factors closely, it is clear that the majority of them encode for proteins that are able to actively suppress both innate immunity and the development T helper 1 (Th1) immune response. For example, vaccinia virus has adopted at least 3 different genes whose product can block the function of the interferon (IFN) family members IFN $\alpha/\beta,\gamma$ (65–67). These factors are secreted by a variety of cells in response to innate danger signals. They can induce an antiviral state and up-regulate adaptive immune functions. Vaccinia also carries genes for multiple inhibitors of chemokines, some of the earliest substances produced during the initiation of an immune response (68–70).

Vaccinia encodes for at least 3 factors that can directly block the function of IFN-γ, one of the most potent Th1 cytokines (71–73). In addition, vaccinia encodes for the recently described IL-18-binding protein (IL-18BP) (74–76). IL-18BP is a naturally produced soluble factor that blocks the binding of IL-18 to its cognate receptor. IL-18BP has been shown to be one of the most potent inhibitors to the development of a Th1-biased immune response (77). Vaccinia virus also encodes for several other immunosuppressive factors, including factors to block complement activation, IL-1β soluble receptor, and soluble tumor necrosis factor receptor antagonist (78–81). These observations suggest that subverting the early innate immune response and slowing the development of Th1

Table 2 Vaccinia Gene Products that Inhibit Immune Response

Vaccinia open reading frame	Function
B13R (SPI-2)	Inhibits IL-1β converting enzyme
E3L	Inhibits PKR activation by dsRNA
K3L	Inhibits phosphorylation of eIF2α by PKR
A53R	Soluble TNF receptor
B8R	Soluble IFN-γ receptor
B18R	Soluble IFN-α/β receptor
B29R	Soluble chemokine-binding protein
C3L	Inhibits complement (C4B, C3B)
B5R	Inhibits complement
B16R	Soluble Il-1β receptor
A44L	Steroid synthesis

responses is important for the efficacy of vaccinia therapy (69,82).

Other studies have confirmed the critical role of Th1 response to clearance of vaccinia viral infection. Van den Broek et al. examined the effect of Th1 (IFN-γ, IL-12) and Th2 (IL-4, IL-10) balance in the clearance of vaccinia virus in mice using cytokine knockouts (KOs) (83). Vaccinia viral replication was enhanced in IL-12 and IFN-γ KO mice, with IL-12-/- demonstrating greater susceptibility to infection than IFN-γ-deficient mice. Interestingly, development of antivaccinia CTL was completely abrogated in IL-12 KO mice but remained normal in IFN-γ-/-. In contrast, IL-4- and IL-10-deficient mice showed marked enhancement of vaccinia viral clearance, suggesting that these cytokines naturally suppress the host response to vaccinia. IL-10-/- mice exhibited greater inhibition of viral replication than IL-4-deficient mice. When the effects of each cytokine on vaccinia infection was examined in recombinant viral constructs, local expression of IL-4 showed a much greater inhibition of host responses. In fact, although the absence of IL-10 resulted in improved clearance of vaccinia virus that was mediated by increased levels of IL-6 and IL-1, the local expression of IL-10 had little to no effect on viral clearance. Similarly, Deonarain et al. showed that IFN alpha/beta KO mice demonstrate markedly enhanced susceptibility to vaccinia viral infection (84).

There are several strategies that have been investigated to circumvent the problem of premature immune clearance. First, one could create a virus that is less recognizable by the immune system. This could be accomplished by mutating the viral coat of the vaccinia virus to make it less cross-reactive with antibodies. However, the poxviridae and, in particular, vaccinia virus is antigenically very complex and it is unlikely that 1 or 2 mutations in viral envelope genes could significantly alter antibody recognition. Further, any mutations in the viral envelope may decrease the infectivity of the virus. Another strategy would be to develop other poxviruses that are able to selectively infect and lyse human tumor cells that do not cross-react with vaccinia. Viruses from the Yatapox genus infect monkeys and secondarily have infected monkey caretakers (85). These viruses do not cross-react with vaccinia, yet they cause human disease and replicate in human cells. The yabalike disease virus is under investigation as another replicating poxvirus for tumor-directed gene therapy (86). Avian poxviruses also do not cross-react with vaccinia virus and have become popular expression vectors (87). They do not replicate in human cells and are less efficient vectors overall.

Another approach to circumventing premature clearance of our vaccinia vector is to create a viral recombinant that actively suppresses host cellular immune responses. Several groups have reported that insertion of Th2-like cytokines such as IL-4 or IL-10 into vaccinia virus increases in vivo viral replication and slows host clearance of infection (83,88,89). However, creation of a virus that is not recognized by the immune system obviously creates serious safety concerns for the population as a whole because unforeseen events could lead to a pathogenic virus that is not immunologically cleared.

A third approach to improving in vivo viral replication involves reversible transient host immunosuppression. Because of the growth of knowledge in solid organ transplantation, we now have available multiple immunosuppressive agents that can very precisely target specific pathways of the host immune response. This knowledge, combined with our growing understanding of the immune response to vaccinia virus, should allow us to reversibly slow the immune response to our vector. This will theoretically allow for more efficient in vivo viral replication in the tumor, higher transgene expression, and greater oncolysis. This will be a feasible approach with vaccinia mutants exhibiting tumor selectivity.

VII. SAFETY CONSIDERATIONS

Safety considerations for cancer gene therapy vectors include direct pathogenicity of the virus, toxicity of the therapeutic gene product, genome insertion with risk for malignant transformation and germ line mutations, teratogenesis, and the ability to recombine with endemic virus or spontaneously mutate to form a more virulent pathogen. Because vaccinia is a cytoplasmic virus, the viral DNA does not transport to the nucleus and therefore integration into the genome is very unlikely. In addition, there is no known latent infection with vaccinia virus and all cells infected by the virus will be killed by the virus. In addition, because poxviruses are not endemic in the population, it is extremely unlikely for recombinations to occur in patients between attenuated strains and wild-type strains, which would result in a more virulent virus with world health implications (90). The stability of the virus has already been proven during vaccination as part of the smallpox eradication program, so it is unlikely for spontaneous mutations to occur which would change the pathogenicity.

However, the properties that make it a useful virus for tumor-directed gene therapy also make it potentially dangerous. It replicates efficiently in human cells, and its pathogenicity as a systemically delivered virus in humans is unknown.

The scarification of the skin during vaccination for smallpox results in viral replication in the dermis, pox formation over 5 to 7 days, and an aggressive immune response against the virus, which eliminates the virus and prevents systemic spread. A permanent scar in the skin results from the infection. It is not difficult to imagine that, if such an infection occurred in an organ such as the brain, this could result in a poor outcome. During vaccination for smallpox, some patients with T cell-deficient immune systems suffered progressive systemic infection and death from vaccinia (63). In vaccine trials for HIV patients, deaths have been attributed to systemic viremia in the setting of an immunocompromised host (91). Although intradermal delivery is quite safe for the vaccine strains, more virulent strains such as WR delivered systemically may be more pathogenic. These viruses need to be carefully examined in preclinical toxicology studies prior to human trials.

Vaccinia and other poxviruses have been identified, designed, or treated such that they no longer replicate in human cells, but still efficiently express genes. These include the MVA strain (attenuated by serial passage in chick embryo fibroblasts, until it is no longer replicated in human cells), NYVAC (life cycle blocked prior to DNA replication in nonavian cells), fowlpoxvirus, and entomopoxviruses (92–94). Vaccinia can also be reliably inactivated using UV light and psoralen such that early genes are still expressed, but no cytopathic effect or replication occurs (95). Also, viral mutants can be constructed with deletions in essential genes preventing replication, except in cell lines where the gene is compensated for by stable integration into the genome. Although all these nonreplicating viruses improve the safety profile, they would not be expected to be efficient for the purpose of tumor-directed gene therapy. Any mutations that result in improved tumor specificity and decreased systemic virulence should be considered. An efficient strain of vaccinia virus such as WR may be mutated to inhibit replication in nondividing cells, but maintain efficient replication in tumor cells. This significantly decreases viral pathogenicity, but maintains vaccinia efficiency as a vector. An enzyme/prodrug approach may inhibit viral replication and provide a switch for turning off infection prior to host toxicity. Treatment with 5-fluorocytosine prolongs survival in a model where mice are administered a lethal dose of vaccinia expressing the cytosine deaminase (CD) gene. This was the original intention of ''suicide genes'' and needs to be explored further in vaccinia (96).

Other strategies toward improving the safety of this vector have been described (36,37,49,97,98). One strategy to attenuate the virus has been the deletion of genes required to evade the host immune response (61). As discussed above, vaccinia expresses several proteins that interfere with the host response to viral infection. These include inhibitors of apoptosis such as the serpins B13R, SPI-1, and SPI-2, inhibitors of cytokines such as interferon, interleukins, tumour necrosis factor, and mechanisms to evade complement (vaccinia complement control protein). Deletions of many of these proteins have led to attenuation of the virus. Safety can also be improved by actively improving the host immune response against the vector. Vaccinia engineered to express inflammatory cytokines are rapidly cleared by the host, leading to decreased pathogenicity. IL-2 expressing vaccinia is rapidly cleared by natural killer cells, leading to marked attenuation. IFN-γ expressing vaccinia was also less virulent (99). Another strategy to attenuate the virus has been the deletion of genes required for viral DNA synthesis. Deletion of the vaccinia TK gene or vaccinia growth factor genes renders the virus dependent on the nucleotide pool of the host cell (36,37). A mutant vaccinia virus with both the TK and vaccinia growth factor (VGF) genes were deleted was highly attenuated in nude mice (100).

VIII. CLINICAL EXPERIENCE

A. Smallpox Vaccination

Extensive clinical experience exists with vaccinia virus as a vaccine for the eradication of smallpox. The most common commercial preparation used in the United States was the Wyeth Dryvax (101). It is the only vaccine available today. The virus was produced by infection of live calves by dermal scarification, followed by physical scraping of the skin. Future vaccines will be produced on cell lines, and ongoing trials are comparing strains for safety and efficacy (56). The vaccine is delivered by scarification of the skin. The lyophilized virus is reconstituted and spread on the skin. A scarification needle is then used to penetrate the dermis through the vaccinia coat in multiple places. Effective vaccination is indicated by the development of pustules 6 to 10 days after vaccination. The pustules represent replicating vaccinia within the dermis. Live virus can be recovered from the pustules from days 3 through 14 after vaccination. There is a direct relationship between the intensity and extent of virus multiplication in the skin and the magnitude and duration of antibody response. The immune protection seems to last a lifetime, including both circulating antibodies and memory T cells (102). The current recommendation, however, is to be boosted with vaccinia every 10 years.

Adverse events occurred in about 1250 per million vaccinations, as described above, including vaccinia necrosum, vaccinia-associated encephalitis, and eczema vaccinatum (102). Aggressive dermal replication occurred almost exclusively in patients who were T cell immunodeficient. The majority of deaths occurred in infants who suffered postvaccinal encephalitis. The risk of complications increased with the more virulent strains of virus used in Austria and Denmark. Despite worldwide use of this live virus vaccine, no reported adverse events related to mutation of the virus to a more aggressive phenotype was ever reported. No viral-induced tumor formation has been reported. Overall, the virus is remarkably safe for use in humans, despite controlled viral replication in the skin of a potentially destructive virus.

B. Other Vaccines

After proven success as a vaccine responsible for the elimination of endemic smallpox in the world, the obvious leap toward using vaccinia as a vaccine for other indications was made, and vaccinia was engineered to express antigens from

other infectious agents. Likewise, as tumor antigens were recognized and defined, vaccinia was used as a cancer vaccine. The size of the vector allows for flexibility in engineering, such that immune-enhancing genes and antigen genes can be recombined together into the genome. In general, these approaches do not rely on targeting of any specific tissues and may not require viral replication, rather they are designed to take advantage of the immune stimulatory effects of the complex viral particle and the efficient transcriptional machinery of the virus. For safety considerations, nonreplicating vaccinia mutants were developed. The known inflammatory response to the vector combined with tumor antigens and immunostimulatory molecules holds promise for cancer therapy. The potential seems great, but controversy exists as to whether complex immunogenic viruses such as vaccinia may be less effective as vaccine vectors against proteins foreign to the virus.

Vaccinia virus has been used in multiple clinical trials as vaccines for treatment of a variety of tumors as well as treatment of infectious diseases such as rabies and HIV (Table 3). Vaccinia virus has been delivered as subcutaneous, intramuscular, intratumoral, and intravesical (bladder) injections in clinical immunotherapy trials without significant vector-related toxicity (60,103,104). Doses of up to 10^9 plaque-forming units (pfu) have been delivered safely. Intravenous injection of fowlpox virus has been performed with no significant toxicity; however, this species does not replicate in human cells. No systemic injection of a replicating vaccinia virus has been performed in human trials.

Allogeneic cell lysate vaccines incorporating vaccinia virus have been explored clinically. In these studies, vaccinia was not used as a vector, but as an immunogen. The virus was not replication competent. A phase 3 randomized, double-blind, multi-institutional trial of an allogeneic vaccinia virus-augmented melanoma cell lysate (VMO) vaccine was performed with 250 patients from 11 centers (105). A 10% survival advantage to VMO-treated patients was detected; however, this was not statistically significant. Hersey et al. in Australia also reported an improved survival in patients treated with an allogeneic vaccinia melanoma cell lysate vaccine (105,106). In many of these trials, DTH response and development of antibodies to tumor antigens correlates with disease-free survival. No vaccinia pathogenicity was observed.

Eder et al. reported a phase I trial of vaccinia expressing prostate-specific antigen in prostate cancer patients (108). The virus was delivered intradermally every 4 weeks for 3 doses. No significant toxicities were related to the virus, which was a Wyeth strain. A cutaneous reaction consistent with viral replication was seen in all patients treated with 2.65×10^7 pfu vaccinia or greater. Fourteen of 19 patients continued to demonstrate cutaneous replication after the third dose. Several patients developed T cell immune responses associated with prolonged stabilization of their cancer. Vaccinia expressing carcinoembryonic antigen (CEA) has been studied clinically as a priming vaccine followed by a boost with avipox expressing CEA (109). This regimen consisted of 1×10^7 pfu Wyeth strain vaccinia injected intradermally, and it was well tolerated. Specific T cell immune responses were generated without clinical responses. A good example of the utility of vaccinia as an immune vector is the development of rV-CEA TRICOM by Greiner et al. (110). This vaccinia expresses a triad of costimulatory molecules: B7.1, ICAM-1, and LFA-3, along with CEA for a vaccine against CEA expressing cancers. This vector demonstrated encouraging preclinical results and is now in clinical trials. Numerous other trials are in varying stages of accrual using vaccinia to express different tumor antigens.

C. Replication Selective Vaccinia "Oncolytic Therapy"

The extent of experience with vaccinia over the years and its proven safety record should lead to acceptance of exploration

Table 3 Recombinant Vaccinia Virus Used in Clinical Trials

First author	Vector	Results
Mastrangelo (60)	Vaccinia-GmCSF	Regression of injected lesions
Marshall (109)	Vaccinia-CEA	No clinical response
Mukherjee (114)	Vaccinia-IL-2	No clinical response
Eder (108)	Vaccinia-PSA	Stabilization of PSA levels
Sanda (126)	Vaccinia-PSA	Stabilization of PSA levels
Conry (127)	Vaccinia-CEA	No clinical response
Tsang (128)	Vaccinia-CEA	No clinical response
Graham (129)	HIVAC-le (gp160)	HIV immunity in healthy controls
Adams (130)	Vaccinia-HPV	Responses in cervical cancer
Kanesa-thasan (131)	NYVAC-JEV	Neutralizing Ab to JEV
McClain (132)	Vaccinia-Hantaan virus	Neutralizing Ab to Hantaan virus
McAneny (133)	Vaccinia-CEA	Safe
Borysiewicz (134)	Vaccinia-HPV	HPV specific immunity
Picard (135)	Vaccinia-gp 160	HIV immunity in healthy controls

of this vector in more novel delivery systems in terminal cancer patients. As the field of tumor-directed cancer gene therapy has developed, the study of tumor-selective replicating viruses has become an important endeavor. Because of the known ability of vaccinia to destroy tissue (a complication of smallpox vaccination known as vaccinia necrosom), vaccinia has also been developed as a direct oncolytic virus for cancer therapy. The concept of using replication-competent viruses to selectively destroy tumors is quite appealing. Numerous viruses have been explored as tumor-selective replicating vectors, including adenovirus, herpes simplex virus, reovirus, newcastle disease virus, autonomous parvovirus, and vaccinia virus (111,112). Advantages and disadvantages exist for each of these vectors, and some limitations are common to all vectors.

Vectors are most limited by inefficient replication in vivo, inefficient tumor targeting, and safety concerns. Vaccinia has many characteristics that overcome these limitations:

1. It has a quick, efficient life cycle, forming mature virions in just 6 h after infection;
2. it spreads efficiently cell to cell thus increasing the efficacy of in vivo infection;
3. it has a large genome that can accept over 25 kb of inserted DNA without deletions;
4. vaccinia virus carries its own strong promoters capable of achieving very high levels of transgene expression;
5. it can infect a wide range of human tissues but does not cause any known human disease;
6. there is a large body of knowledge about its biology and extensive experience with it clinically as part of the smallpox vaccination program.

Preclinical development has focused on mutating the WR strain of vaccinia virus to make it replication selective for tumor cells (100,113,114). The WR strain of vaccinia virus appears to be more efficient in vivo than other strains used in vaccination trials. An intradermal injection of 10^6 pfu of a wild-type WR strain of vaccinia in rhesus macaques leads to a necrotic ulcer of 108 cm^2 in diameter in only 8 days, without systemic spread of the virus. This compares with $<$ 1 cm for NYCBH and Wyeth strains (115). This ability to quickly spread, express genes, and destroy tissue to this extent is unique among current vectors in clinical and preclinical development.

We and others have studied the wild-type virus and found that after intravenous injection, the highest amount of virus can be recovered from the tumor, the second highest from the ovary, and minimal to no virus is recovered from other organs (58,100,116,117) (Fig. 4). The natural tropism of this virus to tumor is surprising, and the mechanism of this is not established. This tumor tropism was demonstrated in numerous tumor models, including murine colon cancer and melanoma, rat sarcoma, human colon cancer in nude mice, and rabbit kidney cancer. Historically, smallpox virus was noted to have

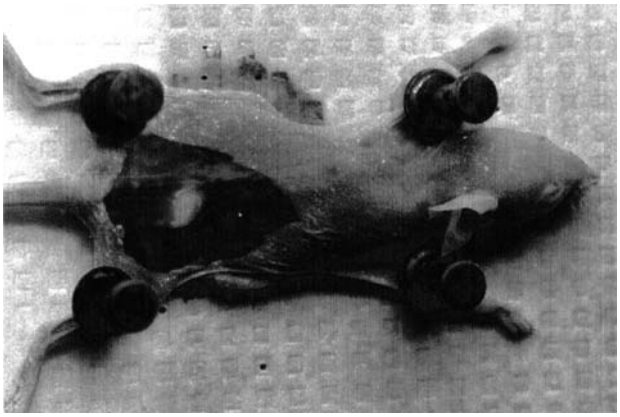

Figure 4 GFP fluorescence is specific for this subcutaneous tumor after intravenous injection of vaccinia-GFP. See the color insert for a color version of this figure.

tropism for injured and irritated skin. This is believed to be secondary to histamine release, leading to leaky vasculature and allowing for transfer of the virus out of the circulation. Vaccinia is a large virus particle (350 nm in diameter) and would require leaky vasculature for extravasation into tissues. Notably, the tumor and ovarian follicles are both known to be sites of vascular endothelial growth factor production and leaky vasculature. As demonstrated by immunohistochemistry, vaccinia tropism to the ovary is specific for ovarian follicles (100).

To develop a tumor-selective vaccinia, the WR strain of vaccinia was mutated such that the VGF and TK genes were deleted (100). The TK gene is important for nucleotide synthesis and DNA replication, and is near essential for replication in nondividing cells where the host nucleotide pool is low. Vaccinia growth factor is a protein that is expressed early by vaccinia virus and is secreted by infected cells. It binds growth factor receptors on surrounding resting cells and stimulates them to proliferate. This increases the available nucleotides in these resting cells, priming them for vaccinia infection. Deleting both the TK and VGF genes leads to near complete abrogation of replication in resting cells, without decreasing the ability of the virus to replicate in the tumor environment. This mutant virus was made and tested, and found to have markedly enhanced tumor specificity (Fig. 5). This virus was tested in rhesus macaques and was found to be completely nonpathogenic when delivered intravenously at doses up to 10^9 pfu (115). Intradermal inoculation at 10^6 pfu demonstrated no viral replication. Nevertheless, 4 days after intravenous virus delivery, equal titers of wild-type and double-deleted virus could be recovered from subcutaneous tumor in mice. This virus can be given systemically at doses of 10^9 pfu to a nude mouse without pathogenicity, and led to marked responses in established subcutaneous tumors (Fig. 6).

One limitation to mutating a virus for selective replication in dividing cells is potential toxicity to other dividing cells in

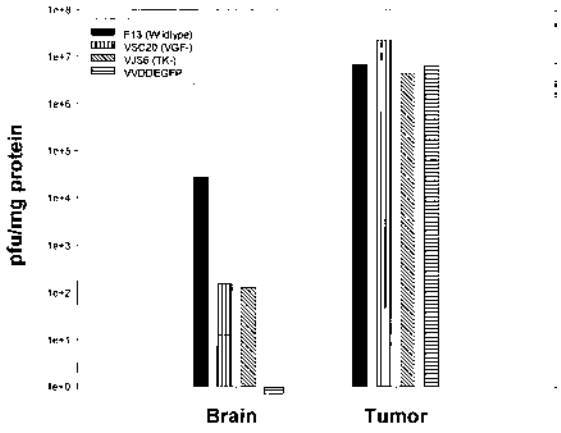

Figure 5 Differential viral recovery (median viral titers) from brain and tumor after systemic injection of 10^7 pfu wild-type, VGF-deleted, TK-deleted, and TK/VGF-deleted vaccinia virus in MC-38 tumor-bearing mice.

vivo. Vaccinia can efficiently infect almost all cell types in vitro, and it is hard to imagine delivering this systemically to animals or humans without significant pathogenicity. Other sites of dividing cells known to suffer toxicity from chemotherapy agents such as bone marrow-derived cells and gastrointestinal mucosa are not affected by systemic vaccinia in murine, rat, rabbit, or primate studies (10). The large virus seems to require a leaky vasculature for extravasation into tissues in addition to proliferating cells. This leaky vasculature is lacking in tissues such as the gastrointestinal mucosa. Despite bone marrow-derived cells having ready access to circulating vaccinia, no bone marrow toxicity is encountered, even

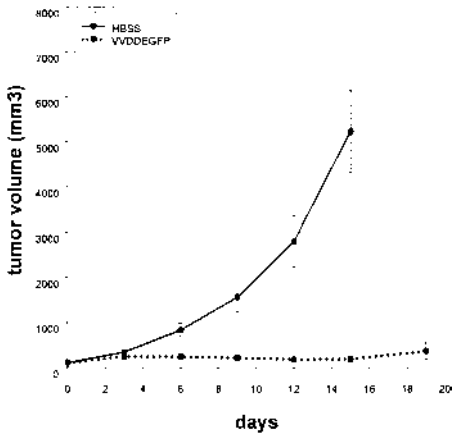

Figure 6 Antitumor response after systemic injection of 10^9 pfu of the TK/VGF-deleted virus in nude mice bearing 5-mm MC-38 tumors. (From Ref. 100.)

in animals succumbing to viral pathogenicity (unpublished observations, 1999). Bone marrow-derived cells are known not to infect well by vaccinia for unknown reasons. The one place that replicating virus is recovered is ovarian follicles. The developing follicle is much like a tumor with developing, leaky vasculature, high levels of VEGF, and replicating cells (118). Immunohistochemistry demonstrates that the double-deleted vaccinia replicates efficiently throughout developing ovarian follicles, without infection or spread through normal ovarian parenchyma.

Although no clinical trials to date have focused directly on the ''oncolytic'' activity of vaccinia virus, intratumoral injections have been explored. In 1974, Roenigk et al. described direct injection of melanoma with vaccinia from standard vialed smallpox vaccine (Wyeth strain) at unknown concentrations in 20 patients at 2-week intervals (119). Numerous interesting antitumor responses of injected lesions were described. Even older studies from the 1960s describe the treatment of warts with direct intralesional vaccinia injection with success (120).

Mastrangelo et al. reported their results of intratumoral injection of a NYCBH vaccinia strain expressing GM-CSF into cutaneous melanoma (60,121). This was a phase I trial of escalating doses up to 2×10^7 pfu per lesion and 8×10^7 pfu per session (multiple lesions injected). Patients were administered twice weekly intratumoral injections over 6 weeks. Systemic toxicity was limited to mild flulike symptoms that resolved within 24 h and local inflammation at the injection site with doses of $\geq 10^7$ pfu per lesion. All patients were vaccinated against vaccinia within weeks prior to receiving the vaccinia-GM-CSF. Interesting responses were seen in 5 of the 7 patients treated. Three patients had mixed responses with complete regression of treated and untreated dermal metastases, 1 patient had a partial response with regression of injected and uninjected regional dermal metastases, and 1 patient with multiple dermal metastases confined to the scalp achieved a complete remission. This group plans to extend their observations with continued clinical trials using this vector.

D. Treatment of Poxvirus Infection

The treatment of orthopoxvirus infections has become of widespread interest recently due to the terroristic threat of biological warfare with smallpox virus. Clinical trials using vaccinia as a gene delivery vector would benefit greatly from a drug or compound that could turn off viral replication. Vaccinia immunoglobulin (VIG) is the only approved product available for treating complications of vaccinia infection. VIG has been owned by the Department of Defense, with a small amount available through the Centers for Disease Control (55). No randomized controlled clinical trials have been performed to evaluate therapeutic efficacy; and prevention therefore, there is doubt as to its effectiveness in established complications from vaccinia. A randomized trial examining comcomitant treatment with vaccinia and VIG demonstrated a significantly lower rate of postvaccinal encephalitis.

Numerous antiviral drugs have been tested in animal models of orthopoxvirus infections (122) (Table 4). Of the

Table 4 Antiviral Compounds Effective In Vivo Against Vaccinia

Animal model	Compound	First author
Vaccinia keratitis in rabbits	Ribavarin	Sidwell (136)
Vaccinia tail lesion formation in mice following IV injection	Interferon	de Clercq (137)
Vaccinia tail lesion formation in mice following IV injection	Polyacrylic acid	de Clercq (137)
Vaccinia tail lesion formation in mice following IV injection	Ara-C, ribavirin, 5-iodo-dUrd, 5-ethyl-dUrd, 5-thiocyano-dUrd	de Clercq (138)
Vaccinia tail lesion formation in mice following IV injection	C-c^3 Ado	de Clercq (139)
Vaccinia tail lesion formation in mice following IV injection	3-Deazaneplanocin A, Ara-A	Tseng (140)
Vaccinia tail lesion formation in mice following IV injection	(S)-HPMPA	de Clercq (141)
Vaccinia related death in SCID mice following IV injection	(S)-HPMPC	Neyts (142)
Vaccinia related death in SCID mice following IV injection	H961 (diacetate ester prodrug of S2242)	Neyts (143)

licensed antiviral compounds, cidofovir has the greatest potential for protection against and treatment of vaccinia infections (123,124). Numerous other agents have also been identified that demonstrate efficacy against different poxviruses. The pharmacokinetics and safety profile of these agents have not been defined in humans, so their true utility will have to be determined. Suicide genes engineered into the virus may function to decrease viral replication upon addition of the prodrug. This has been demonstrated experimentally both in vitro and in vivo using a vaccinia expressing CD followed by the addition of 5-FC (64). Prolonged survival after inoculation with a lethal dose of vaccinia in a murine model was achieved with addition of prodrug compared with controls. Future studies with more potent enzyme/prodrug systems may enhance this effect. Combinations of antiviral drugs and prodrugs may improve outcome.

The development of tightly regulated inducible gene expression systems would allow for in vivo induction of genes, which would be toxic to the virus itself and inhibit viral replication. This has been difficult in vaccinia virus, because of its unique transcription system. Nevertheless, Traktman et al. reported on a tetracycline-inducible expression system in vaccinia (125). This system should be explored further to demonstrate the potential for in vivo induction of genes that are self-toxic.

IX. SUMMARY

Vaccinia virus is an interesting gene expression vector, which is worthy of continued exploration as a gene therapy vector. It is an efficient, destructive virus with some element of baseline tumor specificity. Powerful transcription machinery can lead to very high levels of therapeutic gene expression within tumor cells, and its immunogenicity may lead to an improved immunotherapy application. Mutations lead to tumor-specific replication and direct oncolytic applications. Because the vaccinia virus can include multiple genes, it would be possible to simultaneously express toxic genes, multiple suicide genes, cytokine genes, costimulatory genes, HLA genes, and tumor antigens.

Compared with other replicating vectors such as herpes virus and adenovirus, the study of vaccinia as a tumor-directed vector is in its infancy. Over time the advantages of this vector may become more apparent, and its applicability may be more significant as the population ages and more cancer patients have not been vaccinated against smallpox. Further understanding of the biology of the virus will improve our ability to manipulate it to our advantage and enhance its potential as a vector for tumor-directed gene therapy.

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10

Baculovirus-mediated Gene Transfer: An Evolving New Concept

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I. INTRODUCTION

The great potential of gene therapy has recently been supported by successful treatments of immune deficiency and cancer patients (1,2). However, much remains to be solved before gene therapy becomes a standard procedure. One major obstacle is the low efficiency of gene transfer (3); thus, further development of vectors is necessary. In this context, baculoviruses have raised increasing interest because they have a long history as safe and efficient gene delivery tools in insect cells (4), and the long-lived dogma of incompability with mammalian cells has recently been revised (5–7).

Baculoviruses are common in nature and the food that we consume, and have been known for hundreds of years. Still, no diseases have been linked to baculoviruses in any organism outside the phylum *Arthropoda* (8). Baculoviruses have been studied extensively since the 1950s as biopesticides (9) and, therefore, a lot of data are available about their biology (8) and biosafety (10). The baculovirus expression vector system (BEVS) became a popular choice for recombinant protein production during the late 1980s and 1990s, with a large number of commercially available reagents (4).

It became evident already in early 1980s that baculoviruses can penetrate into nontarget cells, including many human cell lines, but non productively. No viral replication or gene expression could be detected (11,12). However, in 1985, Carbonell et al. (13) reported a successful transduction of mammalian cells by a recombinant baculovirus bearing a promoter active in target cells [Rous sarcoma virus (RSV) long terminal repeat promoter] as part of the recombinant baculovirus genome. In 1995, Hofmann et al. (14) confirmed these results. They were also the first to suggest baculovirus-mediated gene therapy. There was a 10-year gap between these 2 papers probably because Carbonell et al. (15,16) later claimed that their initial findings of low-level gene expression in mammalian cells were due to pseudotransduction. During the late 1990s the concept of baculovirus-mediated gene transfer was further verified and the list of suitable target cells is still continuously increasing.

The fact that baculoviruses are efficiently destroyed by complement (17) delayed the first successful in vivo applications of baculovirus-mediated gene transfer to the beginning of the new millennium (5), although the feasibility of these viruses in an ex vivo perfusion model was reported soon after baculoviruses were suggested as tools for gene therapy (17). Several recent reports have also highlighted the use of baculoviruses in in vivo applications, especially in immune-privileged tissues such as brain or eye (18–20). Indeed, baculoviruses offer many advantages compared with other viral vectors in terms of safety, high capacity for the incorporation of foreign DNA, and easy production (Table 1). This chapter provides a detailed overview of the history as well as current status of baculovirus-mediated gene delivery. Future trends in baculovirus-mediated gene therapy are also discussed. For more detailed background on baculoviruses, the reader can refer to an excellent book, ‘‘The Baculoviruses,’’ edited by Miller (8) and a laboratory manual by O’Reilly et al. (4) de-

Table 1 Properties of Recombinant Baculoviruses that Make Them Suitable for Gene Therapy

Properties	Ref.
Easy to manipulate and produce at high titers ($> 10^{10}$ pfu/mL)	(4,5,18,66,90,106)
High capacity for DNA inserts (>50 kb)	(4,105,106)
Nonreplicative in mammalian cells	(21)
No evidence of viral gene expression	(12–14,140)
Little or low cytotoxicity at high moi	(17,77,80,81,90)
Broad cell-type specificity	(77,80–82,84)

scribing recombinant protein production in insect cells by the BEVS.

II. BACULOVIRUS BIOLOGY

Baculoviruses are a diverse group of rod-shaped viruses having a restricted host range, which is often limited to specific invertebrate species, especially insects (21). Baculoviruses constitute a family of viruses, the *Baculoviridae*, including more than 500 known members (22), which are believed to infect permissively only arthropod hosts. The double-stranded circular DNA genome (80–200 kbp) of baculoviruses (23,24) is condensed into a nucleoprotein structure known as a core (25). The core is located within a flexible rod-shaped capsid, which is 25–50 nm in diameter and 200–320 nm in length (26–28) and can expand relatively freely to accommodate even very large recombinant molecules (29). The core and the capsid are known collectively as a nucleocapsid. The nucleocapsids are made in the nucleus of infected cells, and they acquire membrane envelopes either by budding through the plasma membrane of the cell or by a nuclear envelopment process. Membrane-enveloped nucleocapsids are referred to as virus particles or virions (4).

Baculoviruses are divided into 2 morphologically distinct genera: nuclear polyhedrosis viruses (NPVs), and granulosis viruses (GVs). In the NPV group, virions that obtain an envelope from nuclear membrane are occluded within a paracrystalline protein matrix [occluded viruses, (OVs)], forming large (1–5 μm) polyhedral inclusion bodies (PIBs) containing multiple virions. NPVs are further distinguished on the basis of whether they contain a single nucleocapsid or multiple nucleocapsids per envelope in the polyhedrin matrix (30). In contrast to NPVs, GVs have only a single virion embedded in a very small inclusion body (4).

Baculoviruses have 2 distinct forms involved in their life cycle that play different roles during the natural life cycle of the virus (Fig. 1). OVs are responsible for horizontal transmission between insect hosts, whereas systemic spread within the insect and propagation in tissue culture is dependent on budded viruses (BVs) (8,31,32). Structurally, BVs and OVs differ by the origin and composition of their envelopes (33). They also differ in the mechanisms by which they enter the host cells. BVs enter the cells by adsorptive endocytosis (34), but OVs enter the midgut epithelial cells via direct membrane fusion at the cell surface (35,36). PIBs are formed during late phase of natural infection by embedding the virions in the crystalline protein matrix, which is composed mostly of polyhedrin protein (37). PIBs enable the horizontal infection of larva by contaminating the plant on which the larva feeds (38). The viruses are protected from environmental factors within the PIB, but in the alkaline midgut of the larva the crystalline polyhedrin matrix is solubilized (39) and the released OVs enter the midgut cells by fusion with the membrane of the microvilli (40). During the lytic cycle of infection, the cells release BVs from the basolateral area of the midgut cells (41). The spread of infection within the insect occurs from the midgut to most tissues by hemolymph (42). Eventually the larva dies and the PIBs that are produced in the very late phase of infection are released into the environment, and the cycle begins again. Most of the naturally occurring baculoviruses kill their target host within 4 to 7 days (9).

Baculovirus infection can be divided into early, late, and very late phases. Biologically these phases correspond to reprogramming the cell for virus replication (BV and PIB production). In the early phase (the first 6 h), the virus prepares infected cell for viral DNA replication. This phase is also known as viral synthesis phase. Virus-specific RNAs can be detected in the cells by 30 min postinfection (pi) (43). The late phase extends from 6 h pi to approximately 20 to 24 h pi. During this viral structural phase, late genes are expressed and the production of BVs starts around 12 h pi. Progeny nucleocapsids leave the nucleus and are transported onto the plasma membrane where they acquire their envelope. The occlusion-specific phase begins around 20 h pi. Production of infectious BVs decreases and packaging of virus particles into polyhedrin matrix as OVs begin followed by the cell lysis (4). The packaging into polyhedrin matrix (i.e., production of PIBs) does not take place with BEVS because the polyhedrin gene has been deleted from most baculovirus genomes used in biotechnology procedures.

A. *Autographa californica* Multiple Nuclear Polyhedrosis Virus

The prototype of the family *Baculoviridea* and the most extensively studied NPV-type baculovirus is the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). Its genome (~134 kbp) has been sequenced and predicted to contain 154 open reading frames (44). Although the 3-dimensional structure remains to be determined, the components of its cigar-shaped loosely enveloped virion have been extensively studied by protein chemistry (45). Vp39, p80, and p24 represent the major capsid proteins of the BV form of AcMNPV (Fig. 2). Among them, the vp39 is the most studied and is shown to be randomly distributed over the capsid surface. The major glycoprotein of AcMNPV is gp64, which is the only virus-encoded protein found to be associated with the BV envelope. It is believed to be responsible for the forma-

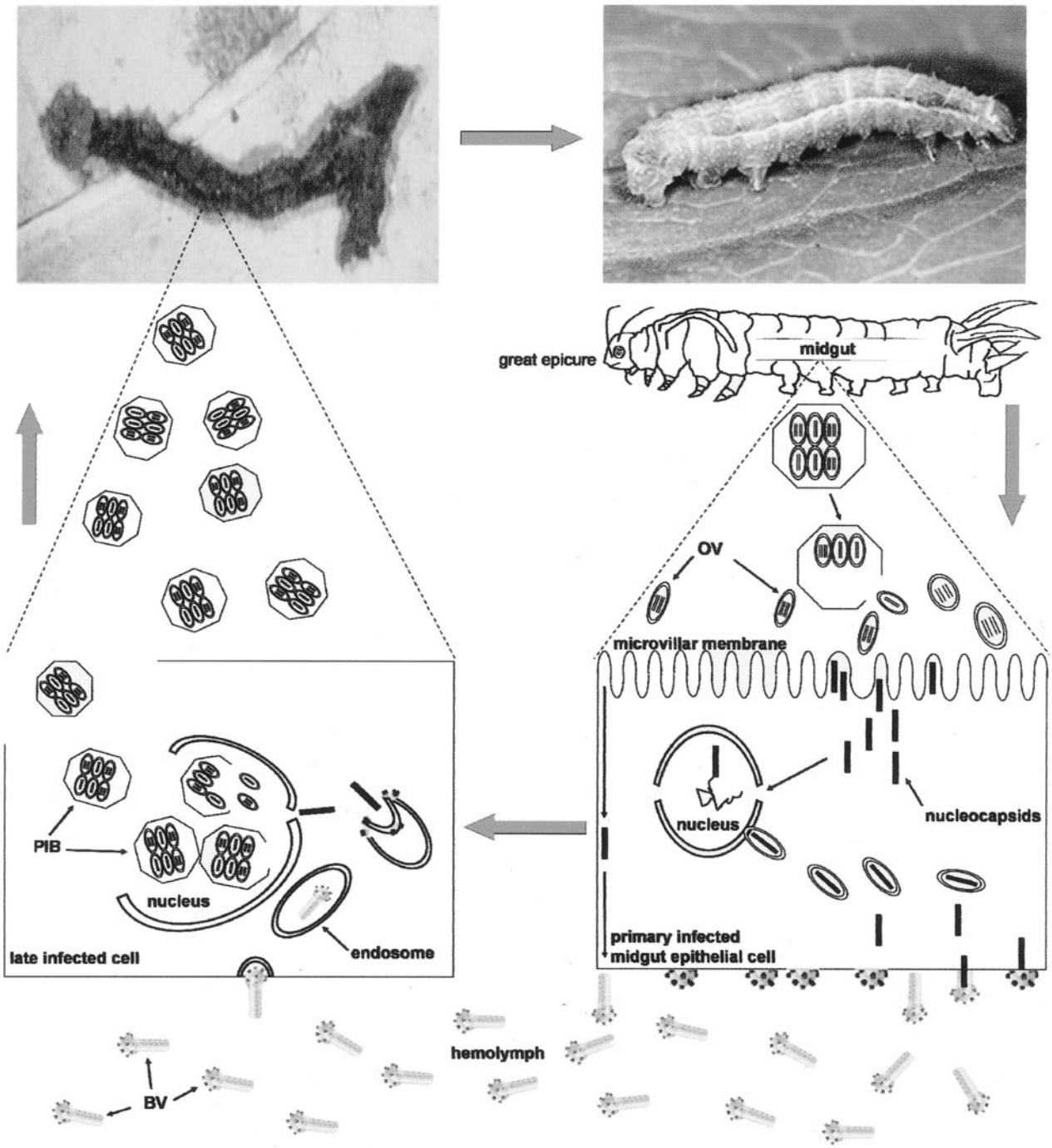

Figure 1 The life cycle of nuclear polyhedrosis virus (AcMNPV). Larva ingests occluded viruses (OVs) containing polyhedral inclusion bodies (PIBs) with their nourishment. In the alkaline environment of the midgut, the PIB break down and the nucleocapsids are released. They infect primarily epithelia of the gut wall. Viruses are fused directly to the plasmalemma of epithelium and the capsids are transported into the nucleus. A minor proportion of viruses may travel through the cells without infecting them in a process called transcytosis. The infection spreads through hemolymph inside the larva via budded viruses (BVs) that contain an essential protein gp64 in their envelopes. Later in the infection, occluded viruses are packed into PIBs inside the nucleus of the infected cells where these particles are released to the environment ready to infect the next hosts (horizontal infection) when cells are lysed and larva dies. , Polyhedral inclusion body; , occluded virus; , budded virus; , gp64 adhesion point in the plasma membrane of infected cells; | nucleocapsid. See the color insert for a color version of this figure.

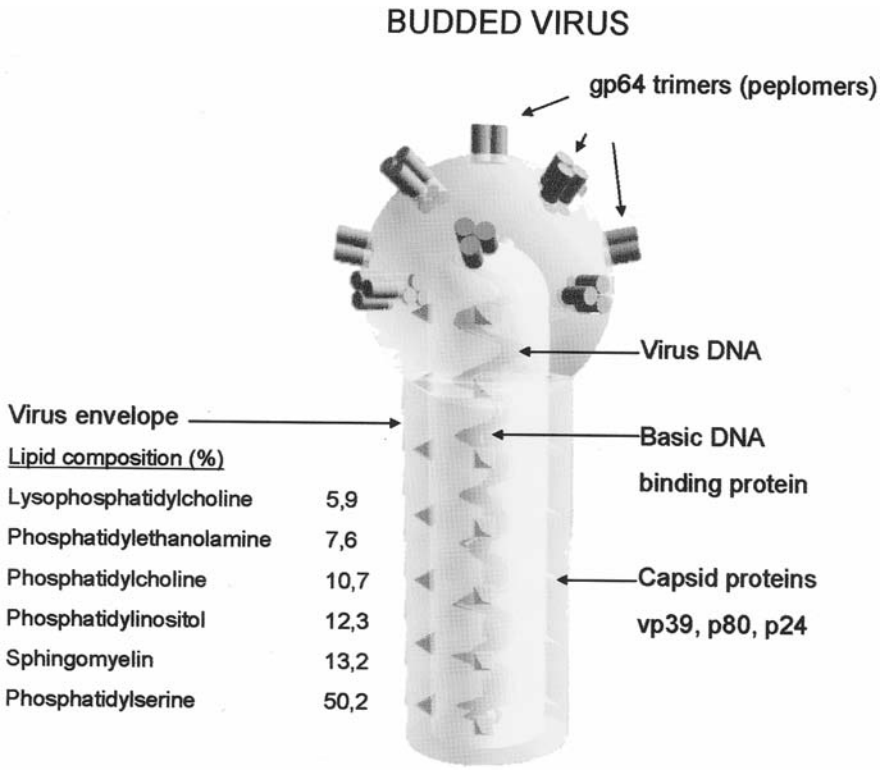

Figure 2 Schematic illustration of the budded form of AcMNPV. (Modified from Ref. 8.) See the color insert for a color version of this figure.

tion of so-called peplomer structure at one end of the virion (Fig. 2). The nucleocapsid of OV and BV are similar, but their envelopes differ in the composition (Fig. 2). The differences in lipid and protein composition of BV and OV envelopes reflect different origin and functions during the virus life cycle (46). Basic DNA-binding protein, p6.9, is shown to be present in the virions of several baculoviruses, including AcMNPV. It may become phosphorylated upon entry into the insect cells, which may result in unpackaging of the viral DNA (47,48).

AcMNPV enters cells by adsorptive endocytosis (34). The role of the major envelope glycoprotein, gp64, is essential for viral entry. It mediates pH-dependent escape of the AcMNPV capsid from the endosomes (49). gp64 is necessary and sufficient for virus preparation because it is needed for efficient virion budding from the insect cells (50). The cellular surface molecules for AcMNPV attachment and entry are not known, but the large range of target cells suggests that the molecules are common cell surface components such as integrins, phospholipids, or heparan sulfate proteoglycans (51–53).

The range of transfer plasmids and parent viruses available for the AcMNPV-based system, and the characteristics (growth, expression level) of the cell lines supporting AcMNPV, have made it a common choice for eukaryotic protein production. The most commonly used cell lines for AcMNPV are Sf-9 and Sf-21AE. They both originate from IPLB-Sf-21 cells, which were derived from *Spodoptera frugiperda* pupal ovarian tissue (54). A new cell line derived from *Trichoplusia ni* egg cell homogenates (BTI-TN-5B1-4 = High Five™), however, has become a popular alternative to Sf cell lines due to the fact that these cells have been shown to produce up to 28-fold more secreted proteins than any other insect cell lines (55–57). These cell lines grow well in both monolayer and suspension cultures, and a serum-free culture medium can be used if desired.

To enable expression of a recombinant protein in insect cells, the gene for the desired protein is usually placed under a strong polyhedrin promoter (*polh*) of AcMNPV (4). For other cells a promoter active in the target cells, such as CMV (cytomegalovirus), RSV, or CAG (chicken β-actin promoter) must be used because *polh* is inactive in these cells. The *polh* is normally responsible for the synthesis of polyhedrin, which can constitute up to 50% of the total protein of the infected cell. Fortunately, polyhedrin is not essential to virus replication or infection in the cell culture (58). However, the use of the *polh* promoter may be restricted in some cases by the fact that it is activated very late in the infection cycle at a point where the host cell machinery for posttranslational modifications is no longer functional. Problems with the *polh* promoter

have been encountered, especially with proteins whose biological activity depends on proper glycosylation (59). In such cases, the use of an alternative strong viral promoter that initiates transcription earlier in the infection cycle (while the host posttranslational modification pathways are still functional) could be useful. Indeed, Chazenbalk and Rapoport (60) were able to produce a more glycosylated and functional form of extracellular domain of the human thyrotropin receptor under a late basic protein promoter (*late*). Other promoters, which will be activated earlier than *polh*, include promoters for the p10 gene (*p10*), the major capsid protein gene (*vp39*), the basic 6.9-kD protein gene (*cor*), and the viral ie1 gene (*ie1*). These promoters are available in a variety of baculovirus plasmids (4,61–63).

B. Preparation of Recombinant Baculoviruses

Due to the large size of baculovirus genome (80 kbp–200 kbp) a homologous recombination procedure was originally adopted to insert foreign genes into baculovirus genome, instead of conventional plasmid cloning techniques (64). In practice, the target gene is subcloned into a transfer vector containing a suitable promoter, flanked by baculovirus DNA derived from a nonessential locus, such as the polyhedrin gene of AcMNPV. The viral DNA and transfer plasmid are then cotransfected into insect cells or yeast cells where the recombination events take place. Typically, 0.1% to 1% of the resulting progeny is recombinant, which complicates their identification. Because the target gene is inserted into the polyhedrin locus, altered plaque morphology of the recombinant viruses can be used for the identification of recombinant viruses. The cells, in which the nuclei do not contain PIBs, contain a recombinant virus. However, the detection of the desired PIB-minus plaque phenotype against the background of greater than 99% wild-type parental viruses is difficult. Viral identification may be facilitated by the introduction of a *lacZ* cassette (β-galactosidase) along with the foreign gene, which enables the detection of the recombinant viruses according to a blue color (4). Drug selection may also be used (65).

Recently, several techniques have been developed to further facilitate and speed up the construction of recombinant baculoviruses (63,66). By using a unique restriction site (Bsu361) at the polyhedrin locus, Kitts and coworkers (67) were able to linearize the double-stranded circular genome of AcMNPV. The linearization reduced the background of wild-type viruses and, as a result, 10% to 25% of the progeny viruses were recombinant. To obtain an even higher proportion of recombinants (85%–99%), Kitts and Possee (68) further modified the AcMNPV genome to enable Bsu361 digestion to also remove an essential gene (ORF 1629) from the AcMNPV genome. Infective viruses will only be reconstituted by recombination with the transfer vector carrying the gene of interest, whereby an intact ORF 1629 will be restored to the genome. The system also enables a blue/white color selection of the recombinant viruses. However, it suffers from the need of time-consuming plaque assays to purify the recombinant virus. A version of this system has been developed in which the target gene is amplified with specific primers using the polymerase chain reaction. This enables the ligase-free coupling of the linearized transfer vector and amplified gene in the mixture, which then can be used directly to transfect insect cells with cut viral DNA. The avoidance of cloning steps in *Escherichia coli* speeds up the construction of the recombinant virus (69). To ease the manipulation of ORF 1629-deleted baculovirus genome, Je et al. (70) recently described a bacmid form of this virus that can be maintained in *E. coli*.

Construction of the recombinant baculovirus genomes by traditional cloning techniques was reported by Ernst and coworkers (71). They introduced the I-*Sce*l meganuclease site into the AcMNPV genome by homologous recombination. The new virus genome, called Ac-omega, can be cut with I-*Sce*l meganuclease, and the target gene bearing compatible ends can be ligated straight to the linearized Ac-omega DNA under a polyhedrin promoter. This method is simple and less time consuming than conventional homologous recombination, but owing to the normal background problems encountered with traditional cloning techniques, the need of plaque purification cannot be entirely avoided. In addition, handling of the large AcMNPV genome (134 kbp) makes the method inconvenient. Another in vitro system based on the preparation of recombinant baculoviruses by versatile Cre-*loxP* recombinase has also been described (72). The advantage of this system is a possibility to recover cloned inserts from baculovirus genome into Cre/*loxP* compatible plasmids. However, only up to 50% of the viral progeny are recombinants.

To avoid laborious and time-consuming plaque purification process, the genetic material can be introduced into the baculovirus genome outside the insect cells. Patel and coworkers (73) reported a novel method of propagating the viral genome by homologous recombination in the yeast *Saccharomyces cerevisae*, where the appropriate recombinants can be more easily selected. Viruses are then obtained by transfecting insect cells. This method is rapid (pure recombinant virus within 10–12 days) and efficient, and it ensures that there is no parental virus background. It also eliminates the need for time-consuming plaque assays, and multiple recombinants can be readily isolated. The major disadvantages of this system are the need of experience in yeast culturing and the incompatibility of traditional transfer vectors with the system. An even faster approach for generating recombinant baculoviruses uses site-specific transposition with Tn7 to insert foreign genes into bacmid DNA (virus genome) propagated in *E. coli* cells. The *E. coli* clones containing recombinant bacmids are selected by color (β-galactosidase), and the DNA purified from a single white colony is used to transfect insect cells (74). The system has the same advantages as the yeast system but is faster (pure recombinant virus within 7–10 days) and easier to work with for those not familiar with the yeast cells. The poor selection features of the original system have been overcome by a modified donor vector (pBV boost) and an improved selection scheme of the baculovirus bacmids in *E. coli* with a mutated lavansucrase gene from *Bacillus amyloliquelaciens*. The new selection schema bypasses the disadvantages

associated with the original transposition-based generation of baculovirus genomes in *E. coli* while retaining the simple, rapid, and convenient virus production (75).

C. Baculoviruses and Nontarget Vertebrate Cells

Baculoviruses have been studied in the past for their ability to infect nontarget cells for safety considerations with regard to their use as biological pesticides. Volkman and Goldsmith demonstrated that baculoviruses were able to enter certain cell lines derived from vertebrate species (12). Thirty-five nontarget host cell lines, 23 of human and 12 of nonhuman vertebrate origin, were exposed to AcMNPV. However, no evidence of viral gene expression was obtained. This study was in accordance with the earlier reports showing uptake of AcMNPV by several vertebrate cell lines with no evidence of viral replication (11,76). Carbonell et al. (13) were the first to show that by constructing a recombinant baculovirus bearing a suitable promoter, marker gene expression in nontarget cells can be detected. Because a low level of marker gene expression was observed in the studied cells, it was claimed that marker protein was carried into the cells with the virus and not actively expressed in the cells (15,16). Several other publications, however, have confirmed Carbonell and coworkers' (13) initial findings and demonstrated that AcMNPVs containing mammalian expression cassettes can enter mammalian cells and express reporter genes under the control of strong viral promoters. Hofmann et al. (14,77) demonstrated that recombinant AcMNPV containing the luciferase gene under a CMV promoter can efficiently infect human hepatocytes (Huh7 and HepG2), as well as primary hepatocytes of human and rabbit origin. Boyce et al. (78) confirmed these results and showed that a virus carrying *lacZ* reporter gene under the control of RSV promoter led to a high-level expression of the marker gene in human hepatocellular carcinoma line HepG2, as well as in primary rat hepatocytes. More recently, primary rat and human hepatic stellate cells (HSCs) were shown to be highly susceptible to baculovirus-mediated gene delivery (79). These findings favor the potential of baculovirus vectors for liver-directed gene therapy.

Further studies have indicated that a high-level expression of marker gene can be achieved not only in hepatic, but also in other cell lines (77,80–84). Shoji et al. (80) transduced a panel of mammalian cells with a baculovirus vector carrying a marker gene in comparison with a replication-defective adenovirus vector. High-level luciferase activity was detected in this study not only in human hepatocytes, but also in other cell lines such as monkey kidney cells (COS7), porcine kidney cells (CPK), and human cervix carcinoma cells (HeLa). Furthermore, the same level of marker gene expression was observed in these cells by both viruses, but as an advantage for the baculovirus, much lower cytotoxicity was associated with it than adenovirus at a high multiplicity of infection (moi). The use of CAG promoter in this study resulted in a 10-fold higher level of luciferace gene activity than in the previous study with a CMV promoter (14). This indicates the importance of the promoter and the expression cassette per se for successful transduction of target cells not only by baculoviruses, but also by any other gene delivery vector (20,85–88). High-level expression of T7 RNA polymerase was also directed by CAG in HepG2 and CPK cells, and lower expression was observed in some other cell lines (81). Condreay et al. (84) demonstrated that recombinant baculoviruses containing green fluorescent protein (GFP) gene under the CMV promoter can transduce a wide range of mammalian cell types originating from different tissues. Cell lines of hepatic origin were transduced efficiently, as described earlier, but notable gene expression was also detected in cell lines derived from kidney tissue (Cos-7, BHK, CV-1, 293) and other nonhepatic hepatic cell lines such as keratinocytes (W12, primary human keratinocytes), bone marrow fibroblasts, and osteosarcoma cells (MG-63). The lowest efficiency of transduction and level of GFP expression was seen in cell lines of hematopoietic origin, such as THP-1, U937, K562, Raw264,7 and P388D1. Efficient gene transfer has also been obtained in primary mouse and human pancreatic islet cells (89). Recombinant baculovirus expressed the glycoprotein gB of pseudorabies virus (PrV) under the CAG promoter in various mammalian cell lines and produced specific antibodies in mice against PrV (90). High levels of expression of PrV gB were observed in many porcine kidney cell lines such as CPK, SK-H, and CPK-NS, and in hamster kidney cells (BHK-21). The potential of baculovirus for therapeutic applications was tested by a virus containing a p53 tumor suppressor gene under the control of the CMV promoter together with an anticancer drug, adriamycin (91). Greater than 95% of Saos-2 cells were killed by the combination of the recombinant virus (moi of 100) and adriamycin (35 ng/mL), suggesting that the combination treatment greatly enhanced apoptosis of the tumor cells. Table 2 summarizes some common cell lines found to be good and poor targets for baculovirus transduction by several independent authors.

Baculoviruses have been pseudotyped by a vesicular stomatitis virus G transmembrane glycoprotein (VSV-G) to further broaden the host range. VSV-G has an extremely broad host range because its entry into the cells seems to not be dependent on the presence of any specific receptor but a phospholipid component of the plasma membrane (92). VSV-G mediates pH-dependent membrane fusion in endosomes (93), and has earlier been used to pseudotype and stabilize other enveloped viruses such as murine retroviruses and lentiviruses (94–96). VSV-G has increased the efficiency of baculovirus transduction in a range of mammalian cells that have been studied (82,97). In these studies, VSV-G was cloned under the control of the *polh* promoter so that it was expressed in infected insect cells but not in mammalian cells. This circumvents the toxicity of VSV-G in mammalian cells. Indeed, the inactivity of baculoviral promoters in mammalian cells is a very useful property of baculovirus vectors. The level of marker gene expression in HepG2 cells treated with a pseudotyped virus was 10-fold higher than in the same cells treated with a wild-type virus. Pieroni et al. (98) showed that VSV-G enhances the transduction efficacy of pseudotyped baculovirus also in vivo. VSV-G can complement the gp64 and there-

Table 2 Common Cell Lines Found to be Good, as Well as Poor Targets for Baculovirus Transduction In Vitro by More Than One Independent Author

Good targets		Poor targets	
Cell lines	Ref.	Cell lines	Ref.
Liver cells		Blood cells	
Huh7	(14,17,20,28,52,77,80–82,84, 97,98,107,112,119,178)	HL-60	(14,77,78)
HepG2	(14,52,77–82,91,97,112,178)		
Primary hepatocytes	(14,77,78,115)		
Kidney cells			
293	(51,78,84,98,106,107,112)		
BHK	(52,84,90)		
COS-7	(20,78,80,112,178)		
CPK 12	(52,80,81,90,178)		
Others			
A594	(14,77,78,82,97)		
CHO	(14,52,82,84,139)		
Hela	(14,20,28,52,78,80–84,97,98, 112–114,178)		

fore allows productive infection, replication, and propagation of the gp64 deleted baculovirus in Sf9 insect cells (99). However, the virus propagation appeared to be inefficient and delayed as compared with the wild-type virus, but virions were similar in morphology to the wild-type viruses. Interestingly, the use of mouse hepatitis virus S protein (MHVS) pseudotyped baculoviruses can also result in a 100- to 500-fold higher marker gene expression than the wild-type virus in many mammalian cells at a low moi (52).

Agents that inhibit histone deacetylation, such as trichostatin A (TSA) and sodium butyrate, can have a significant effect on the gene expression in cells (100,101). The effect is supposed to be mainly due to the more exposed chromatin structure (102). These agents also enhance adenovirus- and retrovirus-mediated transgene expression in vivo and in vitro (5,103,104). Thus, it is not surprising that sodium butyrate and TSA also increase the expression of marker genes significantly in the baculovirus-treated cells in vitro (5,84).

D. Baculovirus Hybrid Vectors

The extraordinary capacity of AcMNPV to carry foreign DNA (no known limit for foreign DNA, but at least 50 kbp tolerated) allows construction of recombinant viruses bearing large expression cassettes (4,105,106). This is a valuable property for many purposes, including hybrid vector construction, as shown by recent reports. Palombo and coworkers (107) constructed a baculovirus-adeno-associated virus (Bac-AAV) hybrid vector to prolong the transient nature of baculovirus-mediated transgene expression. The idea was to use the natural integration capacity of AAV to carry the transgene cassette into a defined region (chromosome 19) of the host cell genome (107). β-galactosidase and hygromycin resistance gene excision from the baculovirus backbone vector and a subsequent integration into the genome of 293 cells was shown to occur with significant frequency. However, integration into the desired region occurred only in a fraction of the clones, and nonspecific integration and multiple insertions of the marker gene were detected.

A system for the production of gutless adenovirus vectors (FD-AdVs) that does not require helper adenoviruses was recently described (106). The helper virus was replaced by the baculovirus-adenovirus hybrid vector (Bac/Ad) containing a Cre recombinase-excisable copy of the packaging-deficient adenovirus genome. 293-Cre cells were transfected with the FD-AdV plasmid containing a transgene cassette, packaging signals, and 2 copies of the inverted terminal repeats, and were followed by transduction by the Bac/Ad. High titer FD-AdV virus preparations (10^8 pfu/mL) were attained. However, the system has to be further improved to avoid generation of replication-competent viruses during a large-scale production.

Yap et al. (81) constructed a recombinant baculovirus carrying a cDNA of the bacteriphage T7 RNA polymerase under the control of the CAG promoter. High-level expression of this enzyme in various mammalian cell lines was observed after the baculovirus transduction. A plasmid bearing the entire poliovirus genome under the T7 promoter yielded a high-titer of infectious poliovirus in the HeLa cells after prior transduction with the baculovirus.

To efficiently propagate and study hepatitis B virus (HBV) and hepatitis C virus (HCV) in cultured cells, recombinant baculoviruses carrying the HBV (108–111) and HCV (105) cDNAs under mammalian promoters have been prepared. The

control of the gene expression in both the HCV minigenome and the full-length HCV construct was also investigated (112). In addition, Tet-off and ecdysome/ponasterone-inducible (pon) systems were compared to control the gene expression. The tetracycline-controlled system gave a low basal activity and was highly inducible in almost 100% of HepG2 cells. Hepatitis-baculovirus hybrid vectors represent a simple and highly flexible system for studying the effects of antivirals and/or cytokines on HBV and HCV production, and for the understanding of their replication and pathogenesis at the molecular level.

To investigate biology of the human cytomegalovirus (HCMV), genes encoding immediate early proteins of the HCMV were cloned into baculoviruses under the CAG promoter (113). These viruses provided a new strategy for efficient isolation of the HCMV viruses with mutations in essential genes.

Finally, a recombinant baculovirus expressing Ebola virus nucleoprotein under the CMV promoter was used to transduce HeLa cells, which were subsequently used as an antigen to detect Ebola virus IgG antibodies from serum samples (114).

E. Entry Mechanisms into the Mammalian Cells

Cell surface receptors responsible for baculovirus attachment and entry are unknown, although the dogma that baculovirus enters the insect cells by an adsorbtive endocytosis has been confirmed by several studies (34). In contrast, the mechanisms involved in the baculovirus entry into mammalian cells are still poorly known. Indeed, several contradictory reports have been published concerning essential cellular motifs responsible for baculovirus uptake into mammalian cells. All these studies, however, agree on that the uptake occurs via an endocytotic route. Early reports suggested that the degree of virus uptake was variable, depending on the cell, incubation time, temperature, and viral phenotype (12). The effect of the titer and virus competition studies in the cell culture have suggested that gene transfer into hepatocytes might be due to the presence of a specific receptor (14). A candidate receptor was postulated to be an asialoglycoprotein receptor. Inhibition tests with choloroquine also indicated that endosomal maturation was essential for virus transport into the nucleus in agreement with the mechanism of virus uptake in the insect cells (Fig. 1) (14,78). Contrary to a supposed specific receptor theory, baculoviruses were reported to enter several cell lines by absorptive endocytosis, which does not require any interaction with a high-affinity receptor but rather interaction with a heterogeneous cell surface motif(s) (51). Electrostatic charges were shown to be important. Neutralization of negatively charged epitopes at the cell membrane appeared to be critical for baculovirus–cell interactions and the subsequent entry. Heparan sulfate proteoglycan seemed to be an important docking motif. Enzymatic removal of heparan sulfate groups from the cell surface caused a significant reduction in transduction (51).

The mode of baculovirus entry into the target mammalian cells has been further studied by constructing recombinant baculoviruses expressing gp64, VSV-G, MHVS, or GFP to compare susceptibility of various cell lines to these recombinant baculoviruses (52). Increased amounts of gp64 or foreign envelope protein (VSV-G, MHVS) on the virus surface (envelope) caused higher expression than the control virus in various mammalian cell lines. Furthermore, this study indicated that phospholipids, such as phosphatidic acid or phosphatidylinositol, on the cell surface played an important role in the transduction of mammalian cells by baculovirus, whereas heparin and heparan sulphate did not.

The basolateral surface has shown to be important in baculovirus-mediated transduction when the hepatocytes acquire intercellular junctions and form islands in the cell culture. Disruption of the cell–cell junctions masking the baculovirus motifs by a calcium chelator, ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), significantly improved the transduction efficiency (115). Baculovirus entry into cells or tissues with tight cellular junctions may thus require transient breakage of intercellular linkages to allow viral contact with the basolateral surface.

Van Loo et al. (28) showed that baculoviruses can also transduce nondividing cells and that the mechanism of the viral capsid transport into the nucleus before uncoating is apparently identical to that of insect cells (Fig. 1) (116). Pig kidney cells (Pk1) were arrested in S phase with aphidicolin, a reversible blocker of DNA polymerase, to show that the arrested cells can be transduced similarly as untreated mitotic cells. An electron microscopy study of the viral uptake showed that baculoviruses enter mammalian cells via endocytosis and are released into the cytoplasm by an acid-induced fusion of the viral envelope with the endosomal membrane in accordance to the entry into the insect cells. Cytochalasin D, which causes reversible depolymerization of actin filaments, inhibited marker gene expression, suggesting that actin filaments are important for viral capsid transport into the nucleus. Similar results were recently obtained also in HepG2 cells and it was further suggested that the transduction block in the nonsusceptible vertebrate cells lies in the cytoplasmic transport or nuclear entry of the virus capsid (117).

F. Gene Delivery In Vivo

Current evidence suggests that baculoviruses also provide an effective tool for in vivo gene delivery. The first attempts of in vivo gene transfer with baculovirus were performed into the liver parenchyma of rats and mice (17). Several attempts were also undertaken to inject baculoviruses directly into systemic and intraportal circulation (17). These experiments resulted in undetectable transgene expression, suggesting that the virus was somehow inactivated by serum components. This led to several studies in immune-compromised animals. When Hofmann et al. (77) injected a β-galactosidase expressing baculovirus directly into the liver parenchyma of C5-deficient immunocompromised mice, a few transduced hepato-

cytes were detected around the injection site. They also injected recombinant baculoviruses into the Huh7-derived human hepatocarcinomas generated in nude mice (T cell deficient) and got low gene transfer efficiency. A systemic baculovirus gene vector delivery into complement deficient Neuro2a tumor-bearing A/J mice resulted in transgene expression primarily in liver, spleen, and kidney, but significant expression was also found in the tumor (118). Injection of decay acceleration factor (DAF)-modified complement-resistant baculovirus vector into the liver parenchyma of complement-sufficient neonatal Wistar rats caused an enhanced expression of the marker gene suggesting that generation of complement-resistant vectors could improve the gene transfer efficiency in vivo (119). However, direct intrahepatic injection of the complement resistant AcMNPV-DAF-βgal vector in adult rats resulted only in single positive cells distant from the injection site.

Delivery methods that allow gene transfer in the absence of serum, or to the sites where viruses are not exposed to the complement, have led to more successful experiments in immune-competent animals. BALB/c mice, nude mice, and Sprague-Dawley rats were injected with recombinant baculovirus directly into the striatum of brain (18,20). Marker gene expression was detected in the striatum, the corpus callosum, and the ependymal layer, indicating the ability of baculoviruses to transduce neural cells in vivo. Transduced cells were identified mainly as astrocytes with only a few positive neurones. No difference was detected between the 3 species or between the cobra venom factor CVF (an inhibitor of the complement system) treated and untreated groups, suggesting immune-privileged nature of the brain. Transduction efficiency, tropism, and biodistribution of the baculoviruses after local delivery into the brain have been studied in comparison to adenoviruses also in BDIX and Wistar rats (18). In this study, baculoviruses were found to transduce cuboid epithelium of the choroid plexus cells very efficiently (76% ± 14). A clear difference was observed with the adenovirus vector when injected into the corpus callosum; adenoviruses did not transduce the choroid plexus cells, whereas ventricular ependymal lining and cells in the corpus callosum were transduced with a high efficacy. Injection into the striatum resulted in an effective transduction near the injection site and in the corpus callosum. Both viruses lead to transgene expression in endothelial cells of brain microvessels throughout the forebrain (18).

In a different approach, carotid arteries of New Zealand White rabbits were successfully transduced by recombinant baculoviruses using a collar device. This system allowed gene delivery with minimal exposure to complement (5). Transient expression in the adventitial cells was observed with an efficacy and duration comparable to adenoviruses. Recombinant baculoviruses have also been tested for direct administration into a mouse eye by subretinal injections. A strong expression of the marker gene in retinal pigment epithelial cells was reported by this study (19). Intravitreal injection of the virus resulted in the marker gene expression in the corneal endothelium, lens, retinal pigment epithelial cells, and retina. The ocular tissue contains areas where antigens are not subjected to the complement pathway and therefore makes it a good target for baculovirus-mediated gene therapy.

Direct injection of recombinant baculoviruses into the quadriceps femoris muscle of BALB/c and C57BL6 mice resulted in a transient expression of β-galactosidase. Expression levels were 5- to 10-fold higher when VSV-G pseudotyped baculoviruses were used (98). The authors also used C5-deficient mice where a higher and more sustained gene expression (up to 178 days) was observed than in BALB/c and C57BL6 mice, suggesting a different gene transfer efficiency between the different mouse strains and the importance of the complement activity.

Baculoviruses have also shown be potentially useful as vaccines. Induction of anti-PrV gB antibody was observed in mice that were inoculated intranasally or intramuscularly. Higher levels of antibodies against PrV gB were detected in serum after intramuscular rather than intranasal inoculation (90). In a related study, Lindley et al. (120) reported a novel use of the baculovirus gp64-display system (121) as a rapid method to produce monoclonal antibodies directed against gp64–fusion proteins. This method might also be useful for vaccination with desired antigens, as suggested by Tami et al. (122). Vaccination with either recombinant baculoviruses or infected cells may prove to be a safe alternative for vaccination approaches (123,124). Table 3 summarizes the current data of the baculovirus-mediated gene transfer studies in vivo.

G. Safety

Since the 1950s, extensive safety testing of baculoviruses has been conducted. These studies have revealed that nuclear polyhedrosis viruses are harmless to and unable to replicate in microorganisms, noninsect invertebrate cell lines, vertebrate cells, vertebrates, plants, and nonarthropod invertebrates. These trials have included long-term carcinogenicity and teratogenicity tests, tests in primates, and tests in humans (10). Ten different mammalian species have been studied, including rats, mice, dogs, guinea pigs, monkeys, and humans. In these tests baculoviruses were administered by a variety of routes, including orally, intravenous injection, intracerebral injection, intramuscular injection, and topically. No toxicity, allergic responses, or pathogenicity associated with the baculoviruses were detected (125). The safety of baculoviruses is also underscored by the fact that we are exposed daily to baculovirus particles present in large numbers in our environment and food. Yet no diseases have been linked to baculoviruses (126).

The safety of baculoviruses is secured at several levels of restrictions in baculovirus infectivity (127). The polyhedrin matrix of OV is essential to horizontal transfer of the virus by protecting it from the environment. The alkaline midgut of insects facilitates the dissolution of OV matrix, leading to primary infection of the larva (Fig. 1). Organisms such as birds and mammals that lack such alkaline conditions in their digestive tract or other potential points of entry, such as respiratory tract, are not infected by OV (128–130). Tissue or cell-

type specificity may create the second level of restriction, although the baculovirus host range does not appear to be limited at the point of entry into the target cells (12,52). This is particularly true for AcMNPV. The subsequent steps following the virus entry by adsorbtive endocytosis may be much more important (28,34,117). Indeed, it was recently suggested that the block in the entry of the AcMNPV into the unsusceptible vertebrate cells lies in the defective transport or entry of the nucleocapsid into the nucleus (117). Although escape from the endosomes certainly creates some barriers, it was found that nucleocapsids seemed to enter into the cytoplasm even in the cells in which no marker gene expression was detected. This is in agreement with the known ability of gp64 to mediate pH-dependent membrane fusion in endosomes (49). Finally, if the nuclecapsid reaches the nucleus, strictly guided molecular mechanisms that cover the expression of the baculovirus genome remain in place. Indeed, baculoviruses propagate only in restricted insect cells and are inherently unable to replicate or express their genes in nontarget mammalian cells (6,131,132). This is a great advantage when baculoviruses are used for gene therapy because risks related to the rise of replication-competent viruses during the virus production can be avoided. Replication-competent viruses are the major concern with most of the current main stream gene delivery vectors based on natural human pathogens (133).

The large size of the baculovirus genome raises a theoretical concern about the possibility of homologous recombination of its genome (or parts of it) into the target cell genome. In the worst case, this might lead to malignancy of the target cell as reported recently in the case of naturally integrating retrovirus and AAV vectors (134–138). Integration of AcMNPV genomic fragments into the mammalian genome has shown to take place in vitro in the presence of selection pressure (84,139). In these studies, Chinese hamster ovary (CHO) cells, which were stably transduced by selection pressure with recombinant baculoviruses, expressed GFP at least 25 passages (84). Analysis of the baculovirus-derived DNA indicated that at least 12 kbp of DNA derived from the viral vector had stably integrated into the transduced CHO cells (84). Integration into the CHO genome occurred as small fragments (5–18 kb) via illegitimate recombination (139). Two of the clonal cell lines maintained starting levels of the GFP expression over a 5-month period with and without selection. The 2 remaining clones, however, showed a loss of the marker gene expression. Baculoviruses can thus be used for the preparation of stable cell lines. However, no evidence of Integration of AcMNPV genomic fragments into the target cell genome has been found without concomitant antibiotic selection pressure (140,141).

One of the biggest challenges in gene therapy is the immune response of the host. The host defense mechanisms function both at the cellular level by generating cytotoxic T cells and at the humoral level by generating antibodies against foreign antigens. Cellular immunity eliminates the transduced cells, whereas humoral immunity protects against the repeated administration of the vector (98,142,143). The host may recognize not only the vector and the transgene product, but also the foreign DNA, which makes the vector design even more challenging (144). However, the eye, brain, and reproductive organs possess immune-privileged regions (145) and therefore may provide good targets for the baculovirus-mediated gene therapy (19,20).

As discussed earlier, immune responses against baculoviruses were suggested as a reason for no detectable transgene expression when mice and rats were treated with systemic, intraportal, or direct injections into the liver parenchyma (17). Therefore, the influence of untreated and heat-inactivated serums from different species was tested for baculovirus transduction efficiency. The results indicated that the classical complement (C) cascade inactivates baculoviruses rapidly and the extent of inactivation varies from one species to another (17,146). Incubation of baculoviruses in the serum of either C3- or C4-deficient guinea pigs did not cause any significant neutralization of the baculovirus, suggesting that C3 and C4 components are essential (17,146). In line with these findings, the direct injection of recombinant baculoviruses into C5-deficient mice resulted in a higher and longer-lasting expression of the marker gene than that in mice, which were not complement deficient (98). In ex vivo experiments that excluded the C system, detectable levels of the marker gene expression were found in human liver segments perfused by the baculovirus vectors (17,77). More detailed investigations have demonstrated that the classical pathway of the C system and assembly of the very late C components are essential for the inactivation of the baculovirus in human serum, indicating the presence of IgM or IgG antibodies against baculoviruses (146). These antibodies are most probably part of the innate self/nonself pattern recognition immune system detecting antigens without a known history of immunization (147,148).

A couple of efforts have been taken to protect baculovirus from inactivation by the complement system (149,150). The treatment of human serum with functional blocking agents (CVF, anti-C5 monoclonal antibodies) against the components of the C-cascade increased vector survival significantly in a dose-dependent manner in vitro (146). Soluble complement receptor type 1, a potent inhibitor of both the classical and alternative C pathways, increased baculovirus survival in human serum in a dose-dependent manner (151). A complete baculovirus survival was achieved by the soluble complement receptor at concentration of 100 μg/mL of serum (151). As mentioned earlier, incorporation of the complement-regulatory protein DAF into the viral envelope was also shown to improve gene transfer efficiency in neonatal Wistar rats in vivo (119).

Baculoviruses have been shown to stimulate antiviral activity in mammalian cells by promoting cytokine production. Baculovirus exposure resulted in the activation of TNF-α, IL-1α, and IL-1β expression in the primary hepatocyte cultures and production of interferons (IFNs) in some mammalian cells (152,153). Kupffer cells were present in the primary hepatocyte cultures and were most probably responsible for the cytokine production (152). The IFN-stimulating activity of the baculoviruses required live virus and was not due to the presence of viral RNA, DNA, or bacterial endotoxin (153). The

detailed mechanism for the observed baculovirus-mediated interleukin induction remains to be studied, but the data suggest a unique process involved in the baculovirus-mediated stimulation of the mammalian IFN. In accordance to these studies, baculovirus-mediated periadventitial gene transfer was found to result in mild immune responses (5). Interestingly, however, only a modest microglia response was seen in rat brain after the baculovirus transduction whereas the adenovirus gene transfer led to a strong microglia response (18).

Despite the profound existing knowledge about the baculovirus safety, further studies are still needed before clinical trials with baculovirus vectors can be launched. Particularly the biodistribution of the virus in vivo is still largely unknown and the immune responses in vivo must be studied more carefully. Further investigations are also required to determine whether some of the viral genes are expressed in mammalian cells. The current data strongly suggest that the AvMNPV genome is strictly silent (6,131,132), but some immediate early genes whose promoters are recognized by host RNA polymerases may well prove to be active (154).

III. FUTURE ASPECTS

Strong evidence now exists to support baculovirus-mediated gene delivery in vitro, and there is very little doubt that this concept is also useful for in vivo. The genuine advantages of baculovirus vector (Table 1) certainly make it a potential new player in gene therapy. However, before this, further studies concerning AcMNPV behavior in mammals, particularly biodistribution and inflammatory issues, need to be studied.

An ideal gene therapy vector should transduce only the desired target cells with a high efficacy. Therefore, a strong interest in developing targeted gene delivery vectors has emerged (155–162). As a first step toward targeted baculovirus transduction, Ojala et al. (163) recently described baculovirus vectors displaying either a functional single chain antibody fragment (scFv) specific for the carcinoembryonic antigen or the synthetic IgG-binding domains derived from protein A of *Staphylococcus aureus*. Display of the targeting moieties on the viral surface was achieved through fusion to the N-terminus of gp64 (Fig. 2) (121). Specific binding of the gp64 fusion viruses to mammalian target cells could be demonstrated by fluorescence and confocal microscopy. However, no enhancement of the viral entry or gene transfer into the mammalian cells could be observed by monitoring GFP expression. Indeed, it is well known that a specific ligand–receptor interaction does not necessarily guarantee efficient transduction of the target cells (161). Internalization, escape from endosomes, and transport of the genetic material into the nucleus are required as well (28,164). The fact that baculoviruses seem to not have any blocks in the entry into the cells (11,12,131,141,165), may make baculovirus targeting

Table 3 Summary of Current Data of Baculovirus-mediated Gene Transfer In Vivo

Study	Target animal	Positive organ/tissue	Positive cell types	Notes
Airenne et al. (5)	Rabbits	Blood vessel, adventitia	Fibroblasts, smooth muscle cells	Collar device used for delivery
Haeseleer et al. (19)	Mice	Retina, corneas	Retinal pigment epithelial cells, retinal inner nuclear layer, corneal endothelial cells, ganglion cell layer, muller cells, lens epithelium, photoreceptor cells	Intravitreal and subretinal injection into eye
Hofmann et al. (77)	C-5 deficient mice	Liver	Hepatocytes	
Hüser et al. (119)	Neonatal Wistar rats	Liver parenchyma	Not determined	Did not work in adult rats
Kirchels et al. (118)	Complement-deficient A/J mice	Liver, spleen, kidney, lungs	Not determined	Systematic delivery through tall vein
Lehtolainen et al. (18)	Normal Wistar rat	Hindbrain, forebrain, spleen, heart, lung	Cupoid epithelium of chorolds plexus endothelial cells in brain microvessels	Intracerebral delivery
Pieroni et al. (98)	DBA/2J, BALB/c, and C57BL/6 mice	Guadriceps, femoris muscle	Myofibers	Best expression of β-gal and the longest expression of EPO in DBA/2J
Sarkis et al. (20)	Nude mice, BALB/c mice, and Sprague-Dawley rats	Brain	Striatum: astrocytes, corpus callosum, ependymal layer neuronal cells	Intracerebral delivery

even more challenging. Further knowledge on molecular mechanisms behind cellular entry will create the basis for further engineering of the baculovirus envelope, allowing better possibilities for cell/tissue-specific entrance. Pseudotyping with known viral surface proteins may also be useful to achieve this goal (52,99,166).

Current baculovirus vectors allow only transient transgene expression, which is desirable in the treatment of cancer or cardiovascular disorders (167). However, more prolonged expression is needed for the treatment of genetic diseases (1). As an attempt to reach this goal, AAV baculovirus hybrid vector was described (107). The strategy to combine elements from known integrating viruses (3) and viruses capable of episomal replication (168) may well prove to be useful. In these kind of vectors, the addition of gene expression control by tetracylin-based system will be desirable (169,170). As time progresses, more baculovirus hybrid vectors will appear to ease the preparation of viruses that are difficult to produce at present (81,105,106,108,171).

A novel system was recently developed where the fusion protein (enhanced GFP) is displayed in large quantities on the surface of the baculovirus capsid without compromising the viral titer or functionality (117). The system is based on the production of the desired peptides or proteins as either C-terminally or N-terminally linked fusion proteins with the baculovirus major capsid protein, vp39 (Fig. 2). This system has many advantages as compared with the baculovirus surface display system, based on baculovirus major envelope glycoprotein, gp64 (Fig. 2) (121,172–174). In vp39, no structural motifs have been recognized either for association with molecules within the stromal matter or for capsid assembly (175). Neither is it responsible for infectivity of the virus. This is important because the major restriction of the (baculo)virus surface display is the fact that displayed proteins may easily destroy the infectivity of the virus in insect cells, leading to low or no titer of the produced virus (176). In addition, immunoelectron microscopy has shown that vp39 is uniformly distributed on the surface of the capsid (175). The baculovirus envelope display system allows fusions only to the N-terminal end of the gp64, whereas the results suggest that vp39 allows the tagging of both termini. Because the length of the capsid can extend relatively freely (29), it is reasonable to expect that this system may be compatible with even larger proteins than GFP. If the penetration of the baculovirus into mammalian cells and the release of the viral capsid into the cytoplasm prove to be a general phenomenon, the concept of baculovirus-mediated therapy may be further extended, with the possibility of using baculovirus capsid as a shuttle to transport therapeutic proteins directly into the cells as an alternative to traditional protein transduction strategies (177).

In conclusion, baculoviruses with many advantages provide an alternative to current gene therapy vectors (Table 1). They are especially useful for large expression constructs allowing the use of multigene strategies. Inherent safety, together with the ease and speed of production of these vectors, creates a powerful concept to test different gene delivery constructs also in a high-throughput manner.

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11

Bacteria as Vectors for Gene Therapy of Cancer

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I. INTRODUCTION

Increasing knowledge about molecular characteristics of malignant cells allows the development of gene therapeutic strategies against a variety of human tumors. Diverse approaches have been followed that can be classified into strategies directly aimed at the tumor or tumor cells, as well as strategies aimed toward the immune system of the host. Due to the successive accumulation of gene defects during tumor development, therapies based on direct gene transfer into tumor cells are mainly of destructive rather than of corrective nature. Suicide killing of tumor cells can be achieved by genetically modifying the malignant cells to express bacterial toxins, prodrug activating enzymes, or tumor suppressors (1,2). The expression of such therapeutics has to be highly restricted to the tumor cells to prevent damage of normal tissues. This selectivity can be achieved by the combination of tumor-targeted gene delivery with tumor-targeted gene transcription. Although transcriptional targeting systems for different tumors have been developed during recent years, tumor-restricted gene delivery is still an obstacle that has to be overcome (1,2).

However, tumor destruction via gene therapy is not exclusively dependent on direct gene transfer into the malignant cells, it can also be achieved by genetic strategies directed toward the immune system of the tumor-bearing host. The motivation of such immunotherapeutical approaches is based on findings, mainly obtained during treatment of melanoma patients, which clearly indicated that the immune system can specifically attack the tumor. Spontaneous regression of primary malignancies can be observed. In addition, tumor-infiltrating T lymphocytes that have the capability to specifically recognize and destroy autologous tumor cells can be isolated. A multitude of studies characterizing the immunogenic nature of tumors and immune responses of the host against it, led to the following concept of T cell-dependent antitumor immunity. Tumor cell destruction can be mediated by cytotoxic $CD8^+$ T lymphocytes (CTLs), specifically recognizing epitopes derived from tumor antigens presented by MHC class I molecules on the surface of the malignant cell. CTL effector activity is dependent on helper functions of antigen-specific $CD4^+$ T cells suggesting that an effective antitumor immune response essentially requires cell-mediated adaptive immunity of a Th1-like phenotype (3,4). Based on this model, immunization protocols are developed that trigger the immune system to overcome the hurdles of immunological tolerance against tumor antigens or escape mechanisms of the tumor. In this context, genetic vaccination might prove great potential. Here, vaccination is attained by administering eukaryotic expression plasmid encoding tumor antigens and/or immune stimulatory molecules in order to activate a cellular immune response directed against the malignant cells.

Irrespective of whether the genetic material is targeted to the immune system or to the tumor or tumor cells, efficient DNA delivery is an absolute requirement. Recently, bacteria have been discovered as an efficient tool for gene delivery to mammalian cells (5–7). This chapter focuses on 2 bacterial delivery systems, *Listeria monocytogenes* and *Salmonella typhimurium*, which had already proven their potential as carriers for heterologous antigens and are now employed in the

delivery of expression plasmids to host cells in vitro and in vivo. In this chapter, the potential use of these bacteria as vector systems for tumor gene therapy is highlighted.

II. BACTERIA-MEDIATED DNA TRANSFER INTO MAMMALIAN CELLS

During infection, some bacteria have the capability to invade particular cells of the host. Dependent on their infection cycle, these microorganisms might establish themselves in different compartments of the target cell. Invasive bacteria such as *S. typhimurium* arrest in the phagosome, whereas facultative intracellular bacteria such as *L. monocytogenes* escape from the phagosome into the cytosol of the infected cell. Independent of whether the pathogens arrest in the phagocytic vacuole or in the cytosolic compartment, both have been demonstrated to be capable of transferring plasmid DNA to the nucleus of the mammalian host cell and to induce heterologous gene expression in vitro and in vivo (7).

In 1995/1996, bacteria-mediated gene delivery to in vitro cultured cell lines was first described by Sizemore et al., Courvalin et al., and Powell et al., using auxotrophic mutants of *Shigella flexneri* as a vector vehicle (8–10). These studies led to the following concept of bacteria-mediated gene transfer: the microorganisms have to invade the target cell and escape into its cytosol, where the expression plasmid is liberated due to death of the attenuated pathogen and subsequently directed into the host cell nucleus (Fig. 1A). This hypothesis had to be partially revised when invasive auxotrophic *Salmonella* were demonstrated to be capable of DNA transfer in vitro and in vivo (11), indicating that phagosomal escape was not an essential prerequisite for gene delivery (Fig. 1B). Interestingly, efficient DNA transfer by attenuated *Salmonella* seems to be restricted to primary murine/human macrophages and dendritic cells (DCs), the professional inductors of an innate and adaptive immune response, respectively (11–16). Only 1 recent study described a low rate of *Salmonella*-mediated gene delivery to cells of epithelial origin, which could be increased by engineering the bacteria to escape from the phagosome into the cytosol (17). The molecular mechanism responsible for *Salmonella*-mediated DNA transfer in the host cell and its restriction to primary macrophages/DCs remains to be determined. The specific biology of macrophages and DCs as professional antigen-presenting cells (APCs) might be responsible for this phenomenon. Macromolecule delivery from the endocytic vacuole to the cytosol has been described for such cells (18,19). However, it might also be attributed to the secretory machinery of the pathogen (20).

In contrast to *S. typhimurium*, in vitro gene transfer to a broad range of host cells has been described for *L. monocytogenes*, including cell lines of epithelial origin, murine primary macrophages, macrophage-like cell lines, and human DC (16,17,22–25). For *Listeria*, efficient plasmid delivery seems to be absolutely dependent on bacterial escape from the phagosome into the cytosol of the target cell (24,25). Interestingly, the capability to deliver DNA into mammalian cells can also be transferred to noninvasive bacteria. Courvalin et al. (9) demonstrated that an auxotrophic *Escherichia coli* strain modified with virulence genes from *S. flexneri* became invasive and delivered plasmid DNA from the cytosol to the nucleus of the host cell. Less efficient but detectable gene transfer was still achieved when attenuated *E. coli* were engineered to arrest in the phagosome, comparable to *Salmonella* (26). But in contrast to *Salmonella*, plasmid delivery by *E. coli* exhibited a broad host range (26). The data on gene delivery indicate that its outcome and efficiency is determined by both, the bacterial vector species and the host cell type. In general, in vitro DNA transfer seems to predictably occur when the bacteria have access to and liberate the plasmid into the cytosol of the host. To achieve plasmid liberation, different strategies have been followed. Most studies employed variant strains that were auxotrophic for a component that is not supplied by the host cell (8–11). Alternatively, bacteria were engineered to lyse intracellularly by the expression of a bacteriophage autolysin (21) or were simply killed by antibiotic treatment (24,25). However, recent studies indicated that invasive wild-type bacteria of the genera *Shigella* and *Listeria* are also capable of DNA delivery to epithelial cells and macrophages without induction of microbial death (17,25). This transfer might be due to cytosolic lysis of individual bacteria that have been damaged during their residence in the phagolysosome or that have been attacked by bactericidal host effector molecules (e.g., reactive oxygen, nitrogen intermediates, defensins), thus indicating that gene delivery might also occur spontaneously under natural conditions.

In comparison to other DNA delivery systems, like viruses, gene delivery by bacterial vectors is characterized by several advantages: (1) plasmids of almost unlimited size, allowing the combination of several genes on one DNA molecule, can be easily transferred into bacteria; (2) plasmid-carrying microbes can be produced and stored at low cost; (3) bacterial vectors can be applied mucosally via their natural route of infection; (4) for many bacterial species approved metabolically attenuated vaccine strains are available; and (5) bacterial infection can be controlled by common antibiotics as a safe guard, an important aspect with regard to the in vivo application of such microbial vector systems.

III. ENHANCING IMMUNE RESPONSES TOWARD TUMORS BY BACTERIAL INFECTIONS

The history of using bacteria in tumor therapy has a long record. Already more than 100 years ago, bacteria were employed by William Coley. Based on his observations on the coincidence of spontaneous tumor remissions and bacterial infections, he systematically treated cancer patients with viable infectious agents (Coley's toxin). By the application of different bacteria preparations, he could obtain impressive therapeutic effects including the complete regression of inoperable cancers in several patients (27,28). How can this acci-

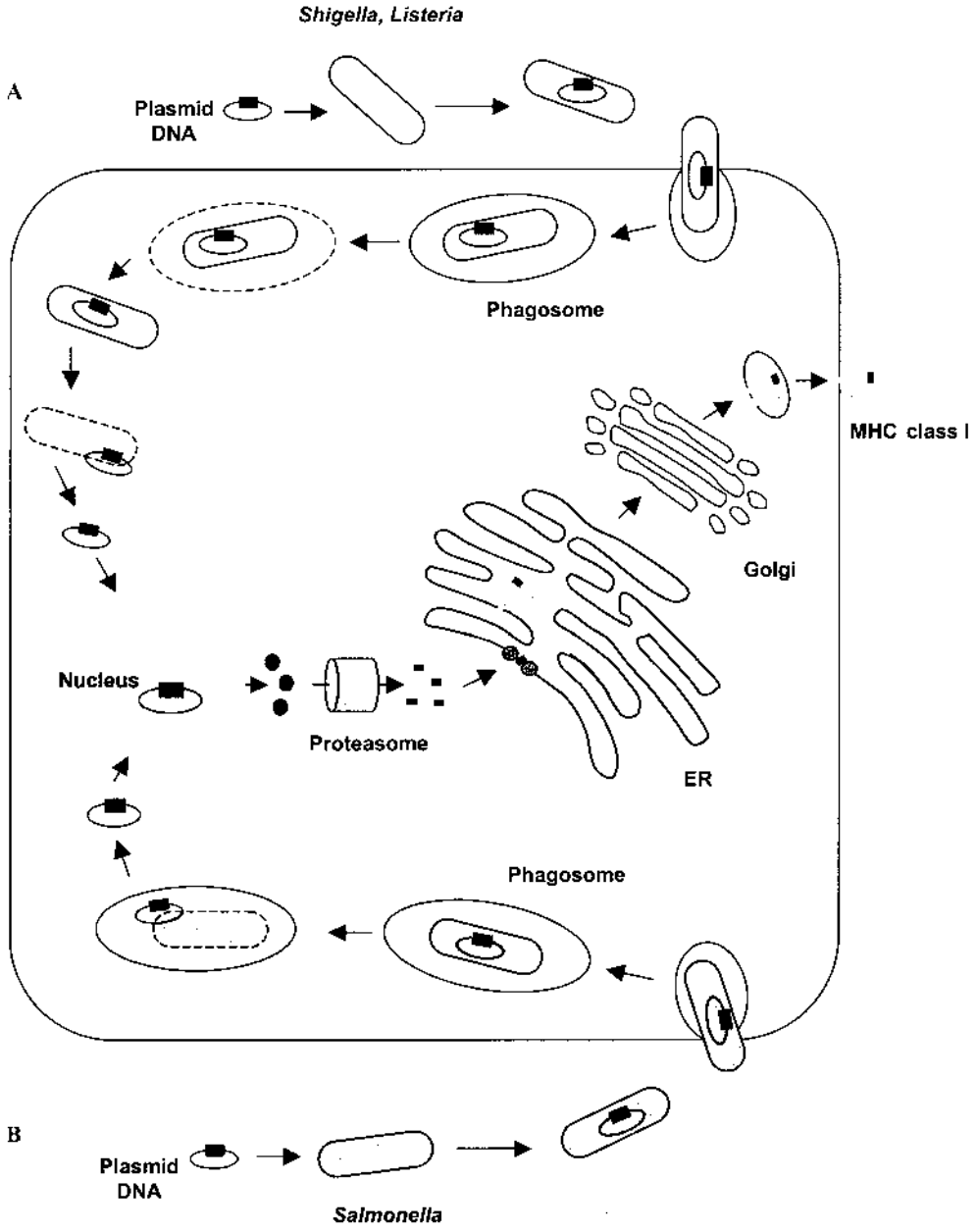

Figure 1 Principles of bacteria-mediated gene transfer. (A) Plasmid DNA encoding the gene of choice is introduced into invasive bacteria such as *Listeria* or *Shigella*. After phagocytic uptake by the target cell, the recombinant bacteria escape from the phagosomal vacuole into the cytosol. The microbes die in the cytosol either due to metabolic auxotrophies, due to genetically engineered autolysins, or by antibiotic treatment. Thereby the plasmid is released, which subsequently obtains access to the nucleus of the infected cells for the foreign gene to be transcribed. The protein antigen is degraded by the proteasome and resulting peptides, containing the appropriate binding motif, are loaded onto MHC class I molecules in the ER (endoplasmatic reticulum). The peptide–MHC class I complex is then transported via the golgi to the cell surface. (B) Facultative intracellular bacteria of the species *Salmonella* are modified with plasmid DNA. The recombinant pathogens invade the host cell and establish themselves in the phagosome. The bacteria die (e.g., due to an auxotrophic attenuation) whereby the plasmid DNA can access the cytosol due to an unknown pathway. Subsequently, the plasmid is transferred to the nucleus of the cell where expression of the gene takes place.

dental finding of a connection between bacterial infections and tumor regressions be explained?

In principle, initiation of antigen-specific immunity is restricted to the lymphoid organs where the target antigens are presented to the T cells by mature dendritic cells. To do so, immature DCs pick up antigens in the periphery. Simultaneous to antigen uptake, DCs have to receive specific environmental signals that induce their activation and maturation. During this maturation process, they obtain migratory capacity toward secondary lymphoid organs and, in addition, become highly stimulatory for T cells by expression of costimulatory molecules. This, leads to antigen-specific T cell activation. With respect to this activation cascade, tumor cells lack the capacity to activate primary T cells, due to the absence of essential stimulatory functions. Although proteins derived from a tumor might be phagocytosed by immature dendritic cells, such DCs normally do not become activated and can therefore not function as inductors of a T cell response. Antigen presentation by immature DC is even considered to result in peripheral tolerance of T cells (Fig. 2). Thus, two opposite functions are attributed to antigen presenting DC depending on their maturation status: they may act as mediators of T cell tolerance or as central players in the induction of adaptive immune responses (29).

How can DC activation be induced? Inflammatory cytokines released in response to tissue damage during infection, as well as highly conserved molecular structures derived from the infectious agent itself, are mediators of DC activation. The latter substances can be considered conserved biochemical patterns that cannot be found in the mammalian host (e.g., unmethylated CpG motifs of bacterial DNA and lipids, such as lipopolysaccharide for Gram negative and lipoteichoic acid for Gram positive bacteria). These molecules bind to Toll-like receptors (TLRs) mainly expressed by cells of the innate immune system and induce their activation (30,31). Several other receptors, such as scavenger receptors, complement receptors, Fc receptors, and the like, might also be involved in the activation process (32). Stimulation via these receptors results in the production of inflammatory cytokines, which in turn might activate additional cell populations, including dendritic cells. Alternatively, DCs themselves can be activated directly by the recognition of pathogen structures through their own pattern recognition receptors. Activated DCs would be the link between the innate immune system and adaptive immunity starting with the activation of T cells of the Th1 type, which might provide essential help for the induction of pathogen-specific CTL (Fig. 2). Thus, the bacterial products are providing strong adjuvant activity that in the context of an antitumor response might be able to break tolerance toward tumor antigens or to circumvent escape mechanisms of the tumor.

One also could envision a scenario where DCs induce a specific immune response against pathogen-derived antigens, while initiating ''bystander activation'' of tumor-specific T cells. When large amounts of tumor antigens are taken up by DCs, this accidental coupling of T cell immunity against microbes and tumor appears to be possible (Fig. 2). This concept might explain the therapeutic effects of Coley's toxin in cancer patients. Nevertheless, despite its effectiveness, this initial immunotherapeutic strategy was ignored for more then half a century, until in the 1960s, when the adjuvant effects of bacterial preparations were rediscovered by the successful use of BCG (*Mycobacterium bovis* strain Calmette-Guerin) in cancer therapy (33).

As a logical consequence, bacteria are now widely used as delivery system for various vaccination strategies, including immunizations against model tumors (34). This was recently extended to genetic vaccination (i.e., bacteria have been used as vehicles for transfer of eukaryotic expression plasmids encoding tumor antigens or immune stimulatory molecules). Obviously, the combination of the adjuvancy of a bacterial carrier and particular features of the antigen expression plasmid should synergize to induce protective antitumor responses. During bacteria-mediated DNA vaccination, DCs might acquire the tumor antigens from the bacterial carrier either by direct infection or via cross-presentation. In the latter case, the infected cell that contains the antigen is phagocytosed by neighboring DCs, which then are activated and represent the original antigens. Both routes, cross-priming and direct priming have been described for specific activation of T cells. In addition to genetic vaccination, bacterial carriers can also be used for gene therapy to improve the performance of the immune system or to directly target inhibitory molecules to the tumor cells.

IV. *L. MONOCYTOGENES* AS DELIVERY VEHICLE FOR EUKARYOTIC EXPRESSION PLASMIDS

The ability of *L. monocytogenes* to transfer eukaryotic expression plasmids into host cells has been mainly explored in vitro so far. However, vast experience exists for the use of such bacteria as a carrier for heterologous antigens, especially in murine tumor systems.

A. L. monocytogenes-mediated DNA Transfer In Vitro

L. monocytogenes infects many cell types in a wide range of different species, including mice and humans. Its intracellular infection cycle, as well as innate and adaptive immune responses against this pathogen in mice, have been extensively studied since the 1970s (35,36). Due to its capacity to induce phagocytosis, *L. monocytogenes* can efficiently infect a variety of cells, including nonphagocytic cells. After its uptake, the bacterium quickly escapes from the phagosome into the cytosol of the host cell, where it replicates. Therefore heterologous plasmid DNA carried by *Listeria* can be delivered directly into the cytosol of the mammalian cell and only has to cross the membrane barrier of the nucleus for expression. Highly efficient plasmid transfer to epithelial cell lines from different species, including fish, mouse, rat, hamster, monkey, and human has been described (16,17,24,25). In addition,

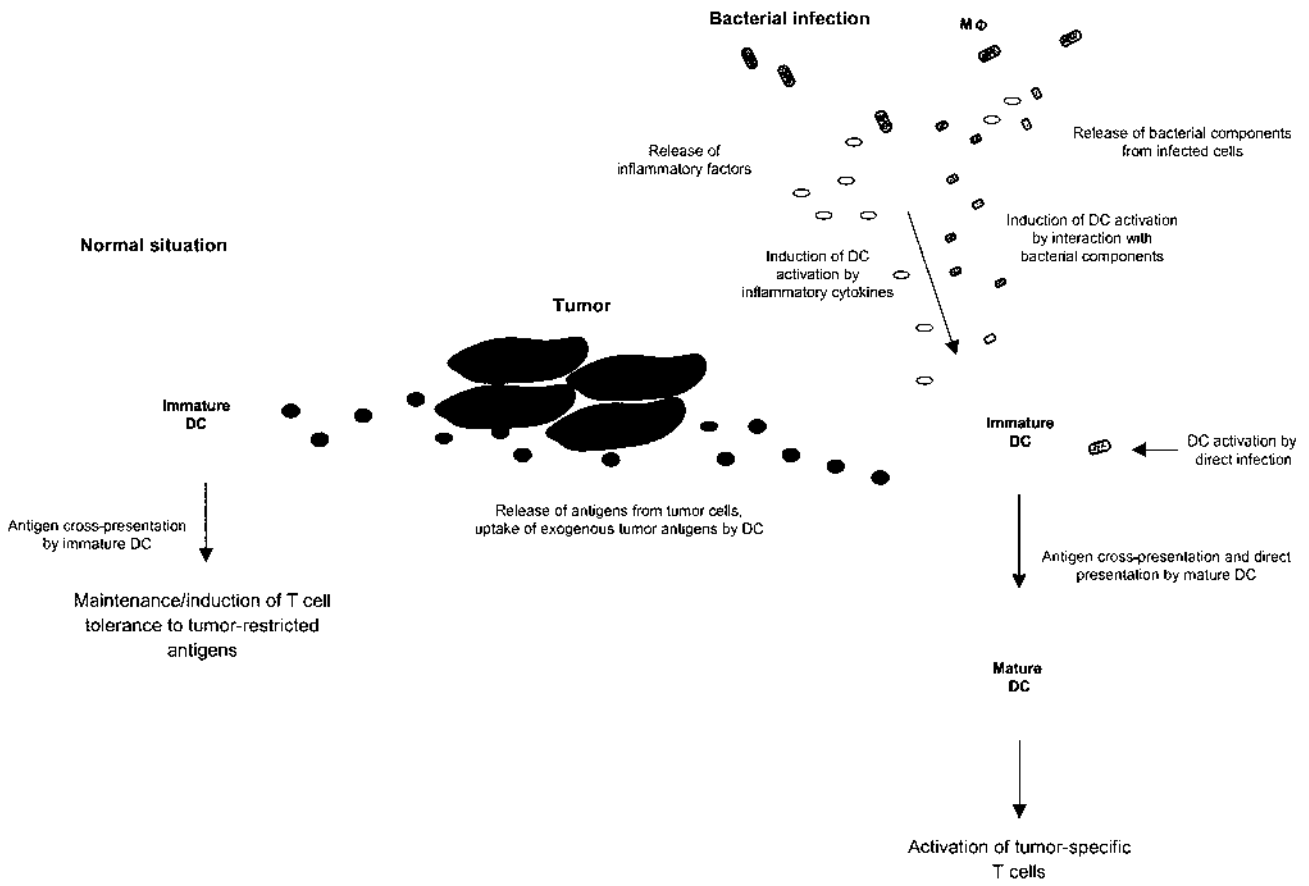

Figure 2 T cell responses to tumors in the presence of bacterial pathogens. Tumors release antigens that are picked up by dendritic cells of the immature phenotype. Presentation of antigens by immature DCs leads to the induction of T cell tolerance. In contrast, bacterial infection is accompanied by the release of inflammatory cytokines, which induce maturation of DCs. In addition, molecules representing conserved pathogen-associated patterns (e.g., unmethylated CpG motifs of bacterial DNA) can be released from infected cells. Alternatively, DCs themselves can be infected and activated. Tumor antigens that are taken up and presented by immature DCs that are activated to mature by the infection will lead to the induction of tumor-specific T cell responses.

professional APC-like murine macrophage-like cell lines, primary macrophages, and human monocyte-derived DCs can be recipients of DNA delivered by *Listeria*, although the frequencies observed vary among studies (17,21–25). This might be due to differences in the experimental settings of infection experiments, but might also be attributed to the mechanism applied to induce bacterial cell death. So far, two strategies have been followed to induce cytosolic plasmid liberation by recombinant *L. monocytogenes*: bacteria were engineered to lyse due to the activity of a bacteriophage autolysin. The expression of this lytic system was under the control of the listerial *actA* promotor and hence was activated preferentially in the cytosol of the host cell (21,23). Alternatively, bacteria were killed by antibiotic treatment of the infected cells (24,25). Interestingly, the way by which an antibiotic interferes with the bacterial metabolism greatly influences the outcome of gene transfer efficiency. Penicillin, an inhibitor of cell wall synthesis, was superior to substances blocking protein synthesis or DNA replication (25). However, gene delivery also occurs using wild-type bacteria without further manipulation, probably as a result of death of the microorganism caused by natural host cell defense activities (17,25).

The efficiency of gene transfer by *L. monocytogenes* is dramatically dependent on the capability of the bacteria to escape from the phagosome into the cytosol of the target cell. Three virulence factors have been demonstrated to be involved in this phagosomal escape—the pore-forming listeriolysin (LLO; encoded by the *hly* gene) and 2 phospholipases (PlcA/PlcB). For some host cells of epithelial origin and for human DC phospholipase activity seems to be sufficient to mediate bacteria entry into the cytosol. In contrast, in the majority of target cells, LLO activity is absolutely required (25,37,38).

However, LLO exhibits severe toxic effects on some cells. Therefore, the bacteria-to-target cell ratios (MOI, multiplicity of infection) normally need to be kept low, which limits the DNA transfer. To overcome this barrier, *Listeria* mutants were constructed that expressed a listeriolysin of reduced cytolytic activity. Indeed, by increasing of the MOI, such strains were able to mediate greatly enhanced plasmid transfer without inducing adverse effects (25).

B. In Vivo Application of *L. monocytogenes* as Vector System

An attenuated strain of *L. monocytogenes*, impaired in intra- and intercellular spreading, has been demonstrated to be capable of plasmid delivery in vivo. Green fluorescent protein (GFP) expressing macrophages could be isolated from cotton rats after intraperitoneal infection with inducible autolyic bacteria that carried a GFP reporter gene plasmid.

Studies exploiting recombinant *Listeria* in gene therapy of cancer have not been published so far, but the therapeutic potential of *Listeria* in tumor therapy was already impressively demonstrated. Wild-type bacteria and attenuated strains of *L. monocytogenes* have been used as an oral vaccine carrier. *Listeria* modified to express β-galactosidase of *E. coli* as surrogate tumor antigen conferred protective immunity in BALB/c mice against a challenge with β-galactosidase (β-gal) expressing fibrosarcoma cells and reduced the growth of established tumors (39).

Even more impressive were the results obtained by the group of Y. Paterson (40,41). In these studies, bacteria were genetically modified with a prokaryotic expression plasmid that integrated into the bacterial chromosome. The plasmid encoded the influenza nucleoprotein (NP) as a model tumor antigen. The NP gene was fused to a deletion variant of the *hly* gene in order to achieve secretion of the LLO-NP gene product. Intraperitoneal (i.p.) immunization of BALB/c mice with LLO-NP expressing *Listeria* conferred complete protection against an otherwise lethal challenge with tumor cells. Colon and renal carcinoma cells that were retrovirally transduced with the NP encoding gene had been used in these studies. In immunized mice, NP-specific $CD8^+$ T cells were identified as main effectors, but $CD4^+$ T cells also contributed to antitumor immunity. The antitumor reactivity was of sufficient potency to even induce regression of macroscopically established tumors (40). In such model systems, tumor protection and regression could also be achieved when LLO-NP expressing bacteria were administered orally (41). Intraperitoneal immunization of C57BL/6 mice with the same bacteria mediated regression of primary tumors and of established lung metastasis by the B16F10 melanoma, when these tumor cells were modified to express the NP gene (42).

Recombinant *Listeria* have also been successfully used to induce protective immunity against viral antigens associated with cancer development. Bacteria, expressing and secreting the E7 protein from the human papilloma virus-16 in the form of an LLO-E7 fusion protein, induced regression of E7-expressing tumors in C57BL/6 mice (43). Similarly, protective and therapeutic immunity against cottontail rabbit papillomavirus induced papillomas, which progress with high frequency to carcinoma, was established when rabbits were immunized with a cocktail of recombinant *L. monocytogenes* expressing and secreting deletion variants of the viral E1 protein (44). Again the efficacy of this carrier system, even in a therapeutical setting, was demonstrated.

Furthermore, recombinant *Listeria* could induce protective immunity against gliomas expressing the nucleoprotein of the lymphocytic choriomeningitis virus (LCMV-NP) as surrogate tumor antigen (45). Subcutaneous administration of bacteria genetically modified to secrete the LCMV-NP conferred antigen-specific $CD8^+$ T cell-dependent protection against a subcutaneous challenge with NP-expressing glioma cells in rats. After tumor rejection, enhanced tumor immunity was observed due to epitope spreading. Such mice were protected against a lethal intracerebral challenge with NP-expressing and parental glioma cells. Parental cells obviously did not express the recombinant surrogate tumor antigen. This indicates that recombinant *Listeria* can initiate antitumor immunity that is even protective within the central nervous system. This anatomical site is considered immunoprivileged (i.e., it is usually not accessible to immunocytes).

Based on these encouraging data an initial clinical safety study of an attenuated *L. monocytogenes* strain in humans has been performed recently (46). Twenty healthy volunteers received orally escalating doses of bacteria that were attenuated by deletions of the *actA* and *plcB* genes. No long-term health sequelae were observed in these studies. This proves the potential applicability of this bacterial carrier system for extended human trials.

V. *SALMONELLA TYPHIMURIUM* AS CARRIER SYSTEM

The gram negative bacterium *S. typhimurium* has been demonstrated to target DNA efficiently to professional antigen presenting cells, whereas transfer in cells of different origin seems to be extremely ineffective (11,17). The original finding that primary macrophages can be transfected to a high degree in vitro using *S. typhimurium* (11) has been further extended. Percentages of transfectants close to 100% have been obtained with murine and human macrophages (13,14). Human dendritic cells have also been shown to be susceptible to transfection by *Salmonella* (16), although the percentage of transfectants obtained was still low. The obvious efficiency of *S. typhimurium* in transferring eukaryotic expression plasmids to APC in vivo and in vitro also has prompted its use for genetic vaccination in tumor systems with extremely promising results in some studies. In addition, the tumor-homing properties of specific attenuated *S. typhimurium* strain variants make them a useful tool for gene delivery to the malignant tissue.

A. Oral DNA Vaccination Against Model Tumors Using Attenuated *S. typhimurium* as Carrier

In a few studies, β-gal of *E. coli* has been used as a surrogate tumor antigen in a prophylactic setting. Partial protective im-

munity could be induced against an aggressive fibrosarcoma by orally administering *Salmonella* carrying a β-gal-encoding plasmid (12). Similarly, using the β-gal expressing murine renal cell carcinoma line RENCA-β-gal, Zoller and Christ have demonstrated superior efficacy in inducing tumor protection when the antigen-encoding plasmids were delivered orally by the *Salmonella* carrier, as opposed to injecting naked DNA intramuscularly (47).

Protective immunity against a challenge with the murine melanoma cell line B16 was achieved using recombinant *Salmonella* for prophylactic vaccination (48). After several administrations of bacteria carrying a plasmid encoding the human gp100 (hgp100) melanoma differentiation antigen, protection of 70% of the mice was observed when challenged with B16 tumor cells transfected with hgp100. Gp100-specific T cells could be detected in the spleen of such mice, and by histology, antigen-expressing dendritic-like cells were found in the mesenteric lymph nodes shortly after intragastric administration of the antitumor vaccine carrier bacteria.

These studies were then extended to autologous tumor antigens. Murine gp100 (mgp100) was used as a tumor antigen in oral *Salmonella*-mediated genetic vaccination. To improve the efficiency of the vaccine mgp100 was fused to the invariant chain, a protein that is intracellularly associated with MHC class II molecules. Therefore, the antigen should be targeted to compartments involved in the MHC class II presentation pathway in order to provide antigen-specific T cell help. In addition, IL-2 was administered subcutaneously around the melanoma location in some of these experiments. The combination of oral DNA vaccination and IL-2 treatment prolonged the survival of the mice and was most effective when the tumor was applied at the same time as the vaccination was started (49). Controversial to the above study, no prophylactic antitumor response could be induced in this work.

The group around R. Reisfeld most consequently developed *Salmonella*-based DNA vaccines against autologous tumor antigens. Extremely promising results were obtained by these experiments. First, they used *Salmonella* that carried minigenes encoding epitopes of the autologous tumor antigens mgp100 and mTRP2 fused to ubiquitin for immunization. This resulted in retardation of growth of the B16 melanoma (15). Because mgp100 and mTRP2 are self-antigens, these results suggest that *Salmonella*-mediated DNA vaccination is able to efficiently break immunological tolerance toward autologous tumor antigens.

These findings were extended to a murine neuroblastoma model. *Salmonella*-mediated DNA vaccination was carried out with a minigene encoding epitopes of tyrosine hydroxylase, an autologous antigen found in this tumor. In addition, the complete protein fused to ubiquitin was tested (50). All recombinant vaccines retarded the growth of subcutaneously applied tumors. The complete protein fused to ubiquitin and the minigene, consisting of several epitopes binding to one of the MHC class I molecules, being the most effective constructs. However, this immunization protocol was not effective against metastases (but see below). Further extension of this work also showed that complete proteins without the ubiquitin fusion partner can be used as antigens. *Salmonella*-mediated DNA vaccination was performed in a mouse that was transgenic for human carcinoembryonic antigen (hCEA), whereby hCEA functioned as a self-antigen. Oral immunization of the transgenic mice with *S. typhimurium* carrying an expression plasmid that encoded the complete hCEA, resulted in the induction of specific cytotoxic T cells (51). In addition, the growth of a colon carcinoma expressing hCEA as tumor antigen was retarded. This response could be strongly improved by additionally administering intravenously IL-2 fused to an antibody that targeted the recombinant cytokine to the tumor. When this combination treatment was applied to a lung metastasis model using the same recombinant hCEA expressing tumors and transgenic mice, most animals remained free of metastases (52).

Similar improvements of protective immune responses were reported by the same group using the original B16 melanoma model described above and the epitopes of mgp100 and mTRP2 fused to ubiquitin as antigens. IL-2 targeted to the tumor was additionally applied as adjuvant shortly after tumor challenge. Targeting of IL-2 to the tumor was achieved via a fusion to an antiganglioside antibody, the antigen of which was found on the tumor. This treatment resulted in complete protection against the melanoma in most mice (53). In these experiments, it could be shown that during the induction phase of the immune response no help from $CD4^+$ T cells was required to induce tumor-specific $CD8^+$ cytotoxic T cells. This is in agreement with the strong adjuvant capacity of the bacterial plasmid carrier.

Additional adjuvant capacity was required for protection against a chemically induced colon adenocarcinoma that expressed the hCEA as transgenic tumor antigen. This was achieved by including CD40 ligand (CD40L) into the vaccine construct. CD40L should support the activation and maturation of antigen-presenting cells. A fusion protein of a trimerizing CD40L was therefore generated with hCEA. Mice transgenic for hCEA were then vaccinated with *S. typhimurium* carrying the CD40L/hCEA fusion construct. The vaccination was also combined with the injection of the IL-2-antibody fusion protein that targets IL-2 to the tumor in vivo. Mice treated this way were completely protected against a tumor challenge by the recombinant adenocarcinoma (54). The application of any of the components of this combination alone only partially protected the mice against the tumor challenge.

An alternative approach to improve vaccination against the neuroblastoma described above was chosen by the same group. Only partial protection against subcutaneous tumors had been observed in this model and no effect was detected against metastases (50,55). The original construct encoding tyrosine hydroxylase fused with ubiquitin was modified to include the posttranscriptional regulatory acting RNA element (PRE) sequence of the woodchuck hepatitis B virus (WPRE). This sequence is known to improve gene expression posttranscriptionally by a still undetermined mechanism. *Salmonella*-mediated DNA vaccination using this construct was very efficient in protecting mice from metastases. Control animals had

uncountable numbers of metastatic foci in their liver when injected intravenously with the neuroblastoma. Vaccination with *Salmonella* carrying the unmodified tyrosine hydroxylase encoding plasmid did not reduce this number, whereas vaccination with bacteria that carried the WPRE modified expression plasmid reduced the number dramatically (55). Only a few mice still exhibited a low number of metastases. Finally, complete protection was achieved when this vaccination protocol was complemented with injections of the tumor-targeted IL-2-antibody fusion protein. This schedule was also most efficient in inducing $CD8^+$ T cells expressing IFN-γ intracellularly and tumor-specific $CD8^+$ T cells. When the mice were treated during the tumor challenge with an antibody that depleted natural killer (NK) cells, protection was completely abolished. It was argued that the IL-2 enhances the activation of $CD8^+$ T cells and NK cells, which then synergize in the protective response against the tumor. Interestingly, when the levels of tyrosine hydroxylase expressed from the unmodified and the WPRE containing plasmid were compared by transfection into COS7 cells, no difference could be observed. This could indicate that in vivo the WPRE sequence has different consequences on expression then in vitro. However, peculiarities of viral sequences could be responsible for such an effect, as has recently been shown for a DNA vaccine that included an alphavirus replicon (56).

Based on the possibility of breaking immunological tolerance by *Salmonella*-mediated DNA vaccination, the Reisfeld group recently attempted to interfere with tumor angiogenesis (57). The rational behind these experiments was that the tumor might be able to escape the immune system by loosing antigens, but it cannot escape the necessity to induce new blood vessels for continuous growth. Therefore, an unmodified eukaryotic expression plasmid was designed that encoded the vascular endothelial growth factor receptor 2 (VEGF-R2). Oral application of this construct using *Salmonella* retarded the growth of various tumors even when the challenge was performed 10 months after vaccination. Similarly, when the primary tumor was removed from mice challenged with D121 small cell Lewis lung carcinoma, the vaccinated mice displayed a low incidence of spontaneous metastases, whereas the controls had a high metastatic score. Even more impressive were the results when vaccination was started 10 days after intravenous administration of CT26. All mice treated therapeutically with the vaccine survived and had only a few small metastatic foci, whereas the controls died between 4 and 5 weeks. Mainly $CD8^+$ T cells were responsible for this protective effect because depletion of such cells resulted in loss of protection. In addition, $CD8^+$ T cells could be found associated with blood vessels in tumors of vaccinated mice and cytotoxicity against cells that expressed VEGF-R2 could also be demonstrated. In vivo assays revealed that angiogenesis in such vaccinated mice was severely impaired. Nevertheless, vaccinated mice were fertile like untreated controls and wound healing was only slightly retarded. Thus, *Salmonella*-mediated oral DNA vaccination has proven its great potential in vaccinations against tumors. It is not only applicable in a prophylactic setting, such as in the case of minimal residual disease, but also acts potentially when tumors or metastases are already established.

In spite of this, one has to bear in mind that the immune system is a double-edged sword. Tinkering with it by immunizing with autologous antigens could induce adverse effects. An example of this was observed in a rat lymphoma system with a splice variant of CD44—CD44v2—as tumor antigen (58). CD44v2 is a self-antigen that it is mainly expressed during fetal development. The lymphoma employed was rendered transgenic for CD44v2. When oral *Salmonella*-mediated DNA vaccination was carried out against CD44v2, cytotoxic and helper T cell responses could be measured. However, upon tumor challenge, the tumor grew faster in vaccinated mice and metastases appeared in the thymus. Histological and cytological analysis revealed expression of the antigen in peritoneal myeloid cells. Surprisingly, however, a few antigen-expressing myeloid cells could also be detected in the thymus. Because CD44 and CD44v2 in particular might be a homing factor for the thymus, this might explain why CD44v2 expressing myeloid cells and CD44v2 expressing tumor cells can be found in the thymus. Unsatisfactorily, however, is the explanation of why only the vaccinated mice exhibited thymic metastases. Tolerance induction against the CD44v2 was observed, at least the specific response initially observed after vaccine administrations declined in rats bearing the tumor. The mechanism of such tolerance induction in immune rats remains unexplained so far.

B. *Salmonella*-mediated Gene Therapy

The obvious efficiency of *S. typhimurium* in transferring eukaryotic expression plasmids to host cells in vivo and in vitro has also prompted its use for applications different than genetic vaccination. Transfection by *Salmonella* was used in vitro to complement a monogenic defect in macrophages from patients with hereditary hemochromatosis by transferring a plasmid encoding the cDNA of the hemochromatosis gene *HFE* (14). In addition, several applications in vivo have been reported. Mice defective in IFN-γ would normally succumb to a challenge with *S. typhimurium* aroA. However, when these bacteria carried an expression plasmid encoding IFN-γ and thus were able to transfer DNA that leads to a complementation of the genetic defect at least in some cells, the mice were able to resist the bacterial challenge (13). Similarly, transfer of IL-12 and GM-CSF encoding cDNA via *S. typhimurium* resulted in measurable levels of the particular recombinant cytokine in the serum of such mice and caused the retardation of subcutaneously applied tumors (59).

In a murine B cell lymphoma model, *Salmonella* were used to transfer a plasmid encoding a soluble form of the human CD40 ligand orally (60). Stimulation of normal APCs with CD40L results in up-regulation of MHC class I and class II as well as costimulatory molecules. In the particular B cell lymphomas employed in these experiments, stimulation via CD40L leads to growth suppression in vitro and in vivo. Oral administration of the recombinant *S. typhimurium* protected mice against a simultaneous challenge with the B cell lym-

phoma. This treatment was still partially effective when the recombinant *Salmonella* were applied 1 week after the challenge and still retarded the tumor growth when administered 2 to 3 weeks after tumor application. CD40L was detectable for several weeks in the serum of such mice. Histological examination demonstrated that, after oral administration of the recombinant *Salmonella*, many cells expressed CD40L in Peyer's patches, whereas only a few such cells could be found in spleen. These findings convincingly demonstrate the high potential of *Salmonella* as carrier for DNA vaccines and gene therapy.

C. Targeting Tumors with Transformed *Salmonella*

An unexpected way of bacteria-mediated gene therapy was discovered recently. This is based on the finding that certain anaerobic bacteria such as *Clostridium beijerinckii* and *Bifidobacterium longum* or facultative anaerobic bacteria such as *S. typhimurium* are able to multiply in tumors after intravenous or even oral application (61–63). This is most likely due to the particular microenviroment supplied within a growing tumor (i.e., low oxygen due to insufficient blood supply and nutrients due to dying cells). Correspondingly, *Clostridia* and *Bifidobacteria* are mainly found in necrotic areas of the tumor, whereas *Salmonella* can be found within the whole tumor. Other organs are usually not infected at all (*Clostridia* and *Bifidobacteria*) or to a much lower degree (*Salmonella*). This tumor specificity of accumulation of such bacteria can be used to carry suicide genes into the tumor, this was most consequently worked out for *S. typhimurium*. First, wild-type bacteria and bacteria that were rendered hyperinvasive by several infection cycles through melanoma cells were made auxotrophic for the purine pathway as attenuation (62). Subsequently, the *mlt* gene that is involved in the lipid biosynthesis was disrupted in such bacteria (64). Thus, an altered lipopolysaccharide is produced and these bacteria no longer induce the secretion of TNF-α in macrophages and human peripheral blood monocytes. Therefore, the risk of toxic shock elicited normally by such bacteria is minimized. Despite this, bacteria of both strains are found to reside preferentially in established tumors as compared with normal tissue. An enrichment ratio between 1400 and 2000 was observed between tumor and liver (64). Using one particular *Salmonella* strain of this series, VNP20009, tumor preference was found for several experimental tumors of mouse or human origin [the latter were tested as xenografts in nude or severe combined immunodeficiency (SCID) mice] and enrichment was also observed in spontaneous mammary gland tumors developing in the c-neu transgenic oncomouse (65).

Finally, a variant strain of VNP20009 was generated, TAPET-CD, carrying the gene for cytosine desaminase that converts 5-fluorocytosine (5-FC), a nontoxic prodrug, to fluorouracil (5-FU), a highly toxic antitumor agent. Mice with established autologous or xenografted tumors were treated with bacteria of this strain, and 3 days later 5-FC was administered (66). Shortly after administration, the 5-FU could only be detected in the tumor and not in other tissues, indicating that the enzymatic activity of cytosine desaminase was almost exclusively restricted to the tumor. In addition, all mice survived the treatment and all the tumors tested regressed almost completely. In contrast, after direct application of 5-FU, the compound was found in serum and all the tissues investigated and several mice died due to its toxic effects. Thus, by using bacteria that carry therapeutic genes and are selected to tumors by the particular conditions the tumor provides, it is possible to target chemotherapeutic drugs to the tumor with little effect on the surrounding tissue. Based on these results, the group of S. Rosenberg initiated a phase I clinical study in which the *S. typhimurium* strain VNP20009 was administered to 25 tumor patients with metastatic disease (67). One renal cell carcinoma and 24 melanoma patients were subdivided into cohorts intravenously receiving escalating doses from 10^6 to 10^9 colony forming units (cfu). Doses of 3×10^8 cfu/m^2 were well tolerated. Bacteria induced a dose-related release of proinflammatory cytokines (e.g., IL1-β, TNF-α, IL-6, IL-12), but focal tumor colonization was only observed in 2 patients without any objective induction of tumor regression. This unfortunate result indicates that additional work has to be done to transfer the tumor-homing properties of the bacterial carrier to the human situation.

VI. CONCLUSION

Within a few years after the discovery of bacteria-mediated gene therapy, the spectrum of its applicability has widened considerable. The results on tumor protection are extremely promising because they are not only restricted to prophylactic vaccination against tumor antigens, but also tumor rejection has been observed when therapeutic treatments were undertaken. Obviously, the strong adjuvant effect of the bacterial carrier allowed the induction of protective immune responses against autologous antigens. In some cases, additional cytokine therapy was required or the expression plasmid needed to be improved. However, this indicates the way for further development to obtain the next generation of vectors and improved strategies. For example, the introduction of a viral element into the expression plasmid that was carried by *Salmonella* enhanced the immune response dramatically—from retarding the growth of subcutaneous applied tumors by the unmodified plasmid to protection of most mice against lung metastasis by the modified plasmid. This was further improved by subtherapeutic doses of a tumor-targeted cytokine. Therefore, it is clear that increasing knowledge on cell interactions and cytokine networks of the immune system, on the one hand, and the improved understanding of the pathogenicity of the bacterial carrier organisms, on the other hand, will soon allow the rational design of even more effective vector systems and treatment protocols.

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12

Polyfunctional Vectors for Gene Delivery

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I. INTRODUCTION

Gene therapy relies on nucleic acid carriers. Viral diversity provides a broad palette of possibilities, thus fulfilling most requirements for gene delivery in clinical situations. However, parallel evolution of viruses and their hosts has made foreign protein particles as well as infected cells effective targets for the immune system. Although the immune response to cells can eventually be turned into therapeutic benefit, immune responses to particles excludes repetitive treatment, which is the only realistic therapeutic approach for a chronic disease. In contrast, artificial carriers can be developed without polypeptide components. They can even be coated with an inert layer, thus escaping most of the immune surveillance. Unfortunately, at best only a few out of a million copies of the gene reach the target cell nucleus. Limited biodistribution and ineffective intracellular trafficking are mainly responsible for this low efficiency.

II. DNA COMPACTION

The routing of a foreign gene to the nucleus of a cell is a complex multistage process that requires a multifunctional vector (Fig. 1). The core of synthetic vectors is invariably a polycation capable of inducing DNA condensation. In effect, conversion of a filiform molecule into a compact particle improves both its chemical stability and physical properties. Plasmid DNA compaction by cationic liposomes or polymers is a quasi-irreversible process that leads to microprecipitates containing hundreds of DNA molecules per particle. For transfection of cells in culture, large complexes are advantageous because they sediment onto the cells. As expected, however, their in vivo transfection properties are weak due (among others) to diffusion hindrance.

Unlike the *poly*cationic species mentioned above, *oligo*cations such as spermine or cationic detergents interact with DNA reversibly. Equilibration ensures comparable sizes for all complexes, and entropy tends to direct the system toward the largest number of condensed DNA particles. As a consequence, each particle will be made of a single plasmid molecule (i.e., the smallest possible particle). Unluckily, the other consequence of reversible binding is that DNA complexes do not withstand dilution or binding to polyanions such as proteoglycans (Fig. 1), and hence cannot be used as DNA vectors (1,2).

A chemical solution to this dilemma was found, based on in situ chemical conversion of the cationic detergent into a cationic lipid (Fig. 2) (3). This 2-step process leading to monomolecular *and* stable DNA particles was validated using cysteine-based cationic detergents as condensing agents, and thiol air oxidation into disulfide as the conversion reaction (4). As shown in Fig. 2, the particles all have the same size (25 nm) that corresponds to the volume of a single molecule of plasmid DNA. Moreover, such particles are capable of moving through an agarose gel in electrophoresis conditions, in contrast to classical cationic lipid/DNA complexes, which remain in the wells. To our surprise, the particles moved even faster than plasmid DNA itself (5) (see also Fig. 5). Improved in vivo diffusion within tissues can thus be expected. Intracellular trafficking may be favored, too, especially as noncondensed plasmid DNA was shown to be immobile in the cytoplasm (6). Finally, the size of the particles remains compatible with active nuclear pore crossing, which may facilitate transfection of postmitotic cells.

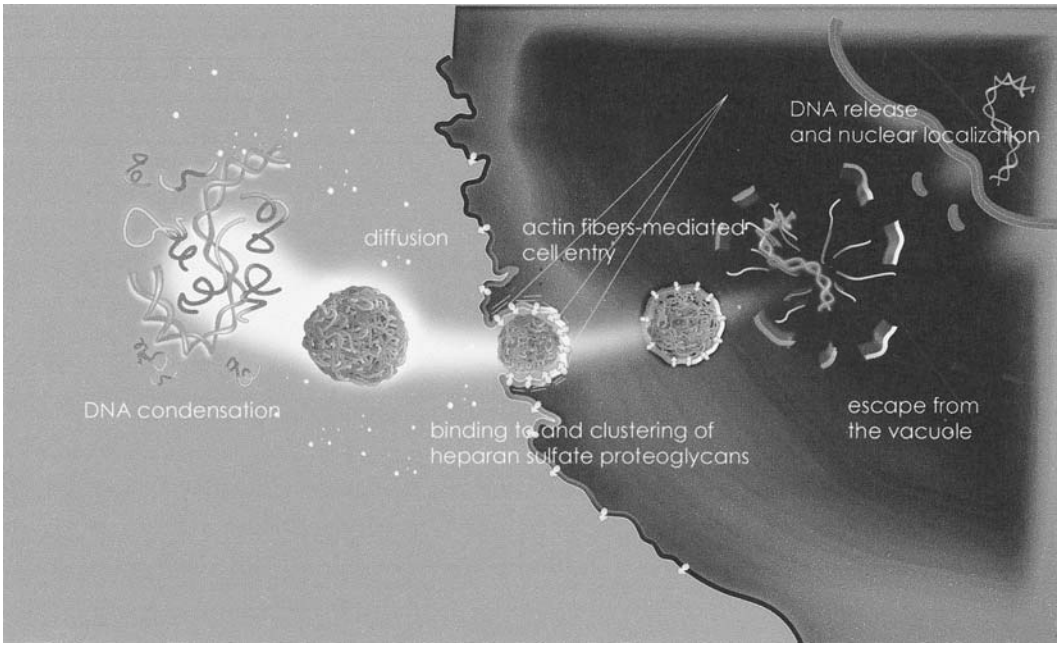

Figure 1 Gene delivery with synthetic vectors is a complex multistep process. See the color insert for a color version of this figure.

III. CELL TARGETING AND ENTRY

To enter their hosts, many viruses and bacteria bind to cell/matrix- or cell/cell-anchoring proteins, such as heparansulfate proteoglycans (HSPGs) and integrins. Anionic HSPGs are involved in cell entry of species with sizes ranging from chlamydia trachomatis (7) to adeno-associated virus (8). Interestingly, condensed DNA particles also require a cationic surface for gene delivery to occur (9), and their cell entry was shown to be mediated by electrostatic interaction with HSPGs (10–12). This is presumably true for all polycationic entities, including calcium phosphate-precipitated DNA.

A common receptor suggests a single mechanism of cell entry. Among ubiquitous HSPGs that may act as receptors are syndecans. This family of transmembrane proteins can cluster to form focal adhesions following individual electrostatic binding to a large polycationic entity. There is evidence (13–15) that syndecans clustering induces their binding to the actin cytoskeleton and eventually the formation of tension fibers. Yet here tension would not provide cell anchoring,

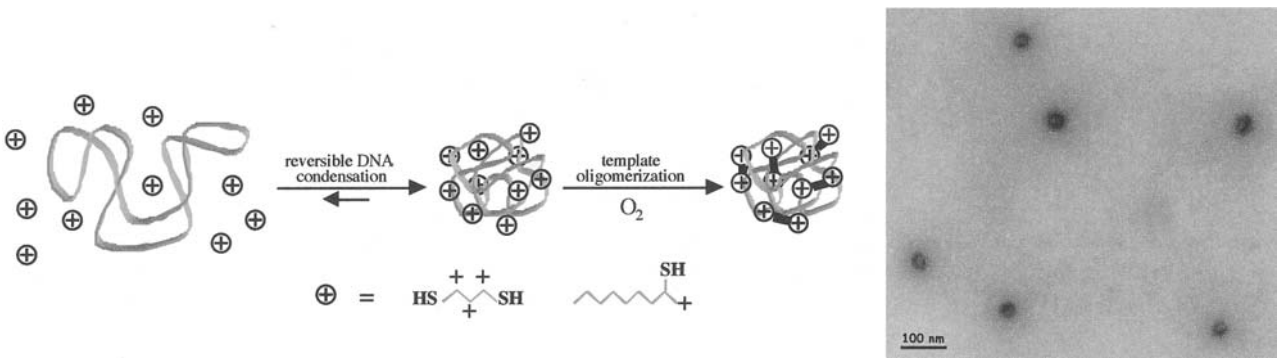

Figure 2 DNA is condensed by thiol-containing oligocations presenting a low binding cooperativity. After equilibration, the particles are ''frozen'' by DNA template-assisted oxidation of thiols into disulfides. Transmission electron microscopy of the particles obtained from a 5.5-kbp plasmid shows a homogeneous population of 25-nm spheres.

but rather a mechanism and the energy to engulf the cationic particle and form an intracellular vacuole (Fig. 1). That cell anchoring and cationic particle binding are competitive processes involving HSPGs may explain why cells have the tendency to detach from their substratum during transfection.

Unfortunately, in vivo a cationic DNA-containing particle will quickly be bound to circulating or extracellular matrix polyanionic proteins. This prevents most particles from reaching their cellular target and eventually releases DNA from the complexes. Therefore, anionic receptors are generally too ubiquitous molecules to serve as targets for synthetic vectors. As mentioned above, integrins share many functional properties with syndecans. Several integrins bind and internalize zwitterionic RGD peptide-presenting particles in vivo (16). The mechanism may be identical to the one described above for HSPGs i.e., mediated by integrin ligation and actin fibers retraction). Imititating adenovirus, RGD peptides have been chemically conjugated to polycations such as polylysine (17,18) or polyethylenimine (PEI) (19,20) and complexed with DNA. The resulting particles were shown to deliver genes to epithelial cells in culture up to 100-fold better than the corresponding polycation–DNA complexes (Fig. 3). Control experiments using RGE-coated particles confirmed the enhanced transfection to be due to $\alpha_v\beta_5$ integrin-mediated cell entry.

IV. STEALTH PARTICLES

An attractive use of RGD-coated DNA particles would be targeting of the neovasculature of metastases following systemic delivery (16). Indeed, resting polarized endothelial cells of normal blood vessels express matrix-binding molecules on their basolateral face, whereas growing and less differentiated blood vessel cells of metastases also express them on the luminal side, hence the targeting. Intravenous injection experiments performed in our laboratory with RGD-PEI–DNA complexes, however, did not target tumors better than the passive accumulation of PEI/DNA or transferrin-PEI–DNA complexes through the leaky vasculature (21). This was presumably due to fast clearance of the complexes. The biodistribution of widely different types of particles has been shown to benefit from coating with a layer of polyethyleneglycol (PEG). This effect is due to the inert nature of the polyether backbone as well as to brush-type polymer crowding, which prevents capture by macrophages. However, the latter property also prevents proper DNA condensation (22,23) and may even interfere with the DNA exchange/release process in the cell. PEI-PEG diblock polymers (22–24–26) or PEG postgrafting strategies (27–29) are being explored as a means to avoid interference with condensation.

As an example of combination of this approach with the monomolecular DNA particle technology discussed above, we currently attempt targeting (30,31) of the high-affinity folic acid receptor, which is overexpressed on many cancer cells. Indeed, folic acid binding was shown to trigger internalization of the complexes (32). To this end, PEG (mw = 3400) was conjugated to a strong DNA-binding moiety one one end, and to folic acid on its distal end (Fig. 4).

Monomolecular plasmid DNA particles were formed with a tetradecane-cysteine-ornithine detergent (C_{14}COrn) (5).

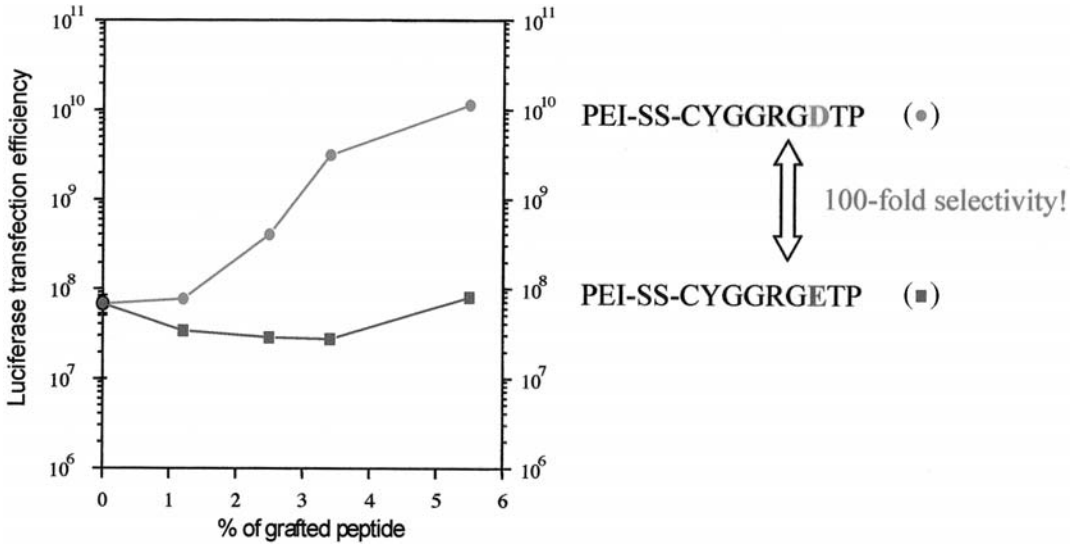

Figure 3 RGD peptide-mediated transfection of HeLa cells expressing $\alpha_v\beta_5$ integrin. Cells were transfected with pCMVluc plasmid complexed to PEI that was grafted with increasing amounts of RGD peptide. Luciferase expression (RLU/mg protein) was measured after 24 h incubation.

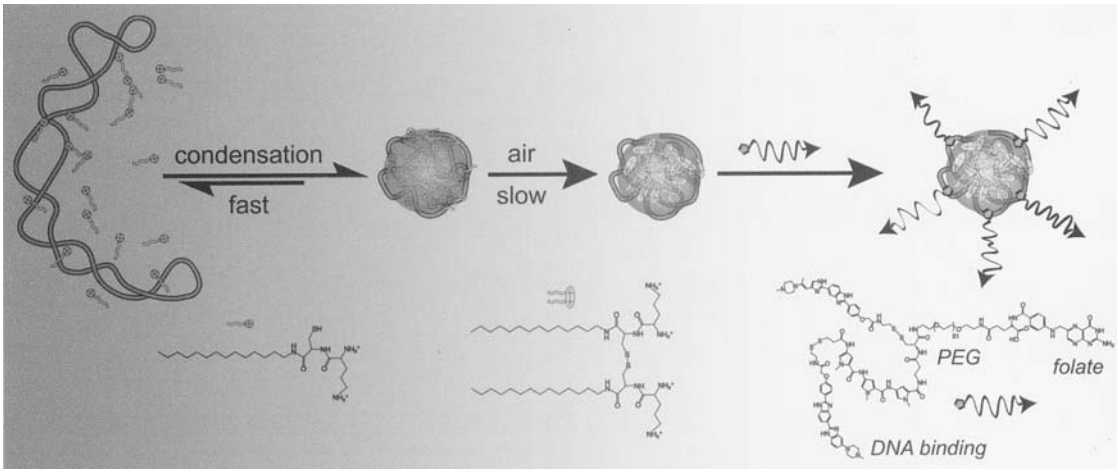

Figure 4 Stepwise assembly of stealth nanometric DNA particles. Plasmid DNA was condensed with a cationic cysteine detergent in aerobic conditions. The resulting gemini lipid/DNA particles were coated with a PEG-folate corona that was anchored to the DNA by a minor groove-binding moiety. See the color insert for a color version of this figure.

Aerobic disulfide bond formation led to stable $(C_{14}COrn)_2$–DNA complexes. Direct binding of PEG-fol to naked DNA as well as PEG-fol grafting onto the surface of the monomolecular DNA particles was monitored by gel electrophoresis (Fig. 5). Electron microscopy showed PEG-coated particles to be compact, monomolecular, and stable in physiological conditions. Finally, cytometry showed them to bind to KB cells (a human nasopharyngeal cancer cell line) when folate receptors are overexpressed (Fig. 6). Targeted gene delivery to cancer cells is being attempted with these complexes.

V. PROTON SPONGE-MEDIATED VACUOLE ESCAPE

After internalization, DNA complexes must escape from the formed intracellular vacuoles (Fig. 1). Many viruses that do not fuse with the plasma membrane exploit endosome acidification as a signal triggering activation of an escape mechanism. This requires sophisticated conformational changes of fusion proteins. In the case of cationic lipids or polymer–DNA complexes, most particles probably remain trapped within vacuoles. DNA being protected within the complexes, this may even be regarded as a ''slow release'' process that sustains gene expression. In effect, cell division, although favoring initial transfection (see below), also leads to loss of the transgene(s) present in the nucleus of a transfected cell. During mitosis of a transfected cell, the intracellular events of gene delivery must thus occur again for that cell to remain transfected, hence the potential importance of slow release. Cationic lipids may possess some intrinsic bilayer-disrupting property, especially when forming nonlamellar phases [e.g., lipopolyamines form direct hexagonal phases (11), DOPE forms an inverted one (33)]. Subsequent vacuole rupture allows charge neutralization of the cationic lipid by intermixing with anionic phosphatidylserine from the vacuole outer leaflet. This may liberate in part the complexed polynucleotide (34).

Cationic polymers possess no fusogenic activity per se. This is why fully cationized polymers such as polylysine require chloroquine, a lysosomotropic drug used to unmask the intravacuolar malaria parasite, to become effective transfection agents. However, some cationic polymers (35), such as polyamine dendrimers (36,37) or PEI(38), share with chloroquine the ability to buffer the acidity of endosomes. Vacuolar pH decrease (39) should therefore coincide with a large ionic concentration increase. Osmotic swelling due to water entry may then burst some of the vacuoles and release the complex into the cytoplasm. This ''proton sponge'' hypothesis (38) proved to be fruitful, as it led to the design of other efficient polymeric vectors (40–42). It was recently supported by the fact that PEI transfection efficiency is 100-fold decreased by bafilomycin A, a specific vacuolar H^+-ATPase inhibitor (43,44).

Over the years, PEI, and especially linear PEI (45–47) has become a versatile vector for in vitro and in vivo gene delivery, with over 300 publications referring to the use of its gene delivery properties. As a leading member of what can be regarded as the second generation of nonviral vectors for gene therapy, in vivo data using PEI deserve a paragraph.

VI. PEI-BASED GENE DELIVERY IN VIVO

As mentioned in the introduction, successful gene delivery and particularly, in vivo delivery, will probably be further optimised with multicomponent approaches. However, despite this caution it is noteworthy that much data on unmodified branched and linear PEIs show them to be efficient, non-

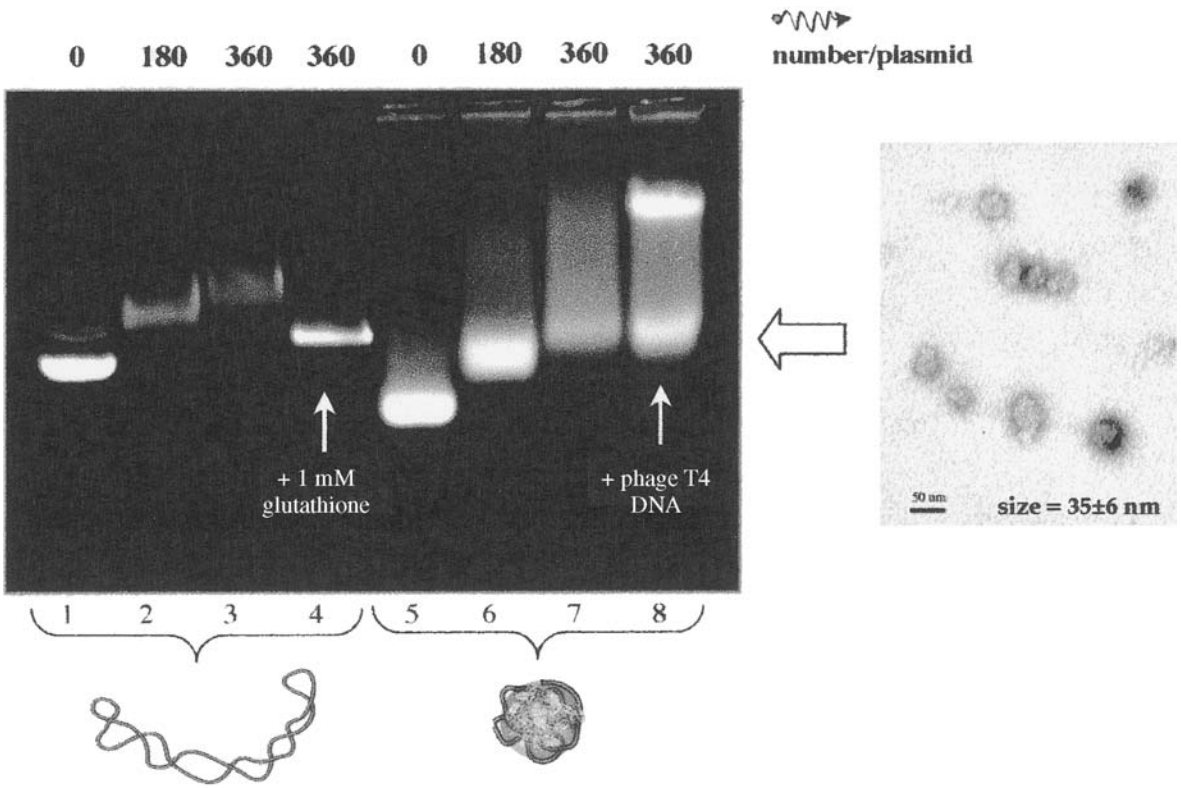

Figure 5 Agarose gel electrophoresis shows complex formation between DNA, cationic detergent, and compound PEG-fol. Plasmid DNA (30 mM bp, lane 1) was mixed with increasing amounts of PEG-fol (1 mM, lane 2 and 2 mM lanes 3, 4); lane 4: DNA–PEG complexes were incubated for 15 min with 1 mM glutation. Lanes 5–8: nanometric $(C_{14}CO)_2$/DNA particles were prepared as in Fig. 4. After overnight incubation, compound PEG-fol (lanes 6–8) was added to the particles; lane 8: 1 h incubation of the complexes with phage T4 DNA. Image: Transmission electron microscopy picture of the final $(C_{14}CO)_2$–DNA–PEG-fol complexes shows monomolecular DNA condensation into compact particles. See the color insert for a color version of this figure.

toxic, and versatile agents for in vivo gene delivery by a number of routes. These commercially available polymers have the added advantage that their relatively high in vivo performance is unhindered by immune effects. Like other synthetic vectors, PEI provides the extra benefit of facility of use for complexing and condensing DNA. This is turn leads to ease of testing DNA constructs and scaling up of protocols. Preclinical data toward gene therapy approaches of AIDS (48), head and neck cancer (49,50), and pancreas (51,52) and ovarian cancer (53) disseminations in the peritoneal cavity are encouraging.

That PEI provides relatively high levels of transfection in a number of target organs, by various delivery routes, prompts a series of questions. Given that the intracellular barriers to delivery are probably similar for in vivo, ex vivo, and in vitro delivery, the main point of inquiry is how DNA–PEI complexes overcome the series of anatomical and physiological barriers that hinder delivery of functional DNA to target cells in vivo. A key point would appear to be the small size of particles obtained when DNA is condensed in a defined medium. PEI–DNA complexes can be formulated that have a size range close to that of viruses (50 nm, Fig. 7) and with a charge ratio that does not hamper biodistribution and yet maintains efficient transfection. According to the choice of formulation solution, small particles can be obtained whether branched (25 kDa) or linear (22 kDa) are chosen. Using linear 22 kDa PEI to complex DNA in salt solutions gives quite large particles ($\geq$1 μm) that work exceedingly well in vitro (47). However, formulation with the same 22 kDa in an equiosmotic but salt-free solution of 5% glucose produces particles with mean size ranging between 30 and 60 nm (54) (Fig. 7). Particles of $<$100 nm with the branched 25-kDa form can be obtained in 150 mM NaCl (23,55). However, despite the roughly similar sizes of particles formulated in appropriate conditions ($<$100 nm), there are quite clear-cut differences in the performances obtained in animal models (47,56) with linear 22-KDa PEI consistently providing the greatest efficiency. Improved transfection of resting cells with linear PEI (46) may be the clue to this observation. The more recent findings on the better performance of the complexes formulated in

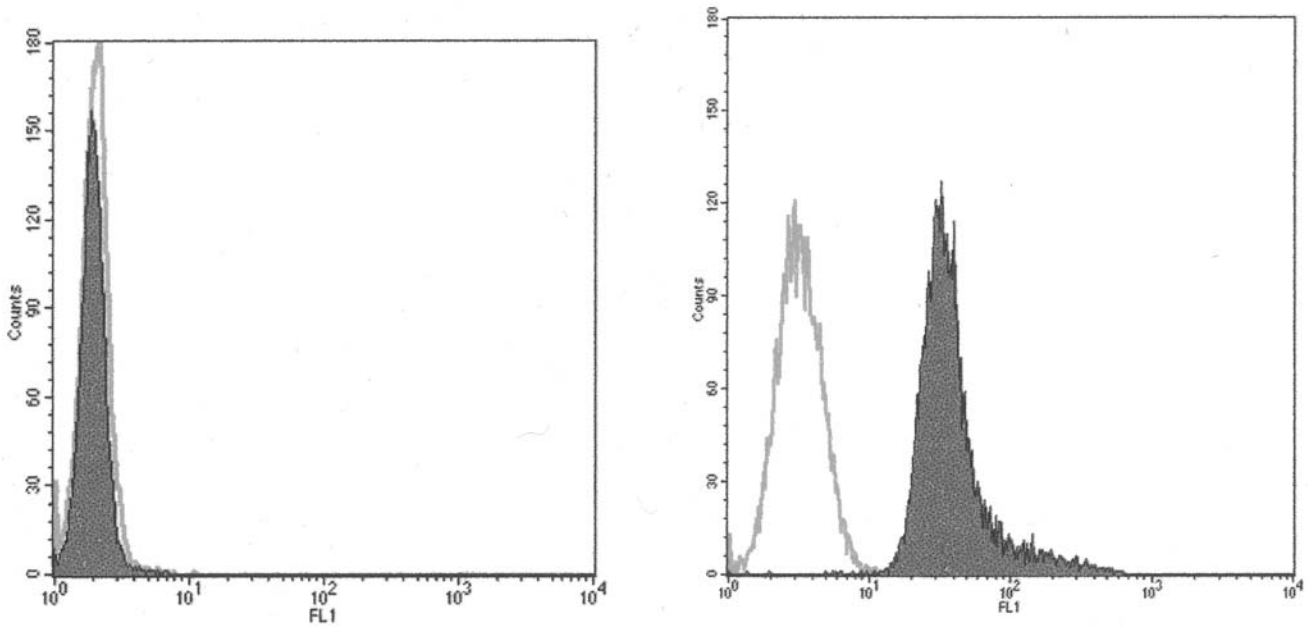

Figure 6 $(C_{14}CO)_2$–DNA–PEG-fol complexes specifically bind to folate receptor-expressing KB cells. Cells were incubated for 3 h with fluorescent DNA complexes and sorted by cytometry according to their fluorescence. Only cells overexpressing the folic acid receptor (right graph) show an increased fluorescence (filled trace) over nontreated cells (hollow trace).

salt-free conditions corroborate early work injecting DNA/PEI particles into the brains of adult mice (57).

The description of the small size of particles formulated in salt-free conditions with 22-KDa PEI was accompanied by the demonstration that these particles were stable in cerebrospinal fluid and led to a large diffusion of active complexes throughout the brain following intraventricular injection (54). This in turn led to the application of the methodology in a number of different settings, including the central nervous system, the respiratory tract, or tumors.

A. Central Nervous System

PEI has been used to deliver DNA into the CNS of rodents both for proof of principle and to analyze physiological functions [for instance, see (58,59)], including regulation of gene expression (promoter studies) and function of proteins (mainly transcription factors and transporter proteins). As concerns the basic optimization of the methodology, keeping concentration of DNA <1 μg/μL increases yield (60). Indeed, in the newborn mouse brain, one can obtain up to 2.10^7 RLU/mg protein when injecting a mere 20 ng DNA. Such conditions also allow for diffusion of particles not only within the intraventricular space, but also through the ependymal cell layer lining the ventricles and through 4 or 5 cells layers beyond it (54). Most recently, using the same intraventricular delivery route, it has been shown that gene delivery with unmodified PEI permits privileged targeting of the neuronal stem cell population in adult mice (61) (Fig. 8).

Similarly, using low DNA concentrations (0.25 μg/μL) and a low charge ratio (3–6 N/P) allowed Marthres and collaborators (62) to obtain increased expression of the dopamine transporter throughout the entire substance nigra (a brain nucleus with a diameter of about 5 mm) in adult rats. Significant increases in binding and uptake were found in all the brain areas receiving neuronal projections from the brain structure transfected (*sustantia nigra*). This finding is important because it underlines the fact that postmitotic neurons can be transfected by this method, in agreement with the related properties of linear PEI (46–63,64). Equivalent results in terms of efficiency of expression in neurons of the adult rat were obtained when injecting plasmids encoding the serotonin transporter into the raphe nucleus of adult rats (65). In this study, effects of modifiying gene expression were also assessed on behavior (sleep/wake cycle), and significant and appropriate changes found for weeks following transfection. Yet another means of gene delivery to the brain with PEI was demonstrated by Wang et al. (66). These authors observed that following injection of PEI–DNA complexes into the tongue muscle of mice, significant reporter gene activity could be found in the brain stem. This finding suggests that PEI–DNA complexes can cross the neuromuscular junction and enter the brain by retrograde axonal transport, thus bypassing the blood–brain

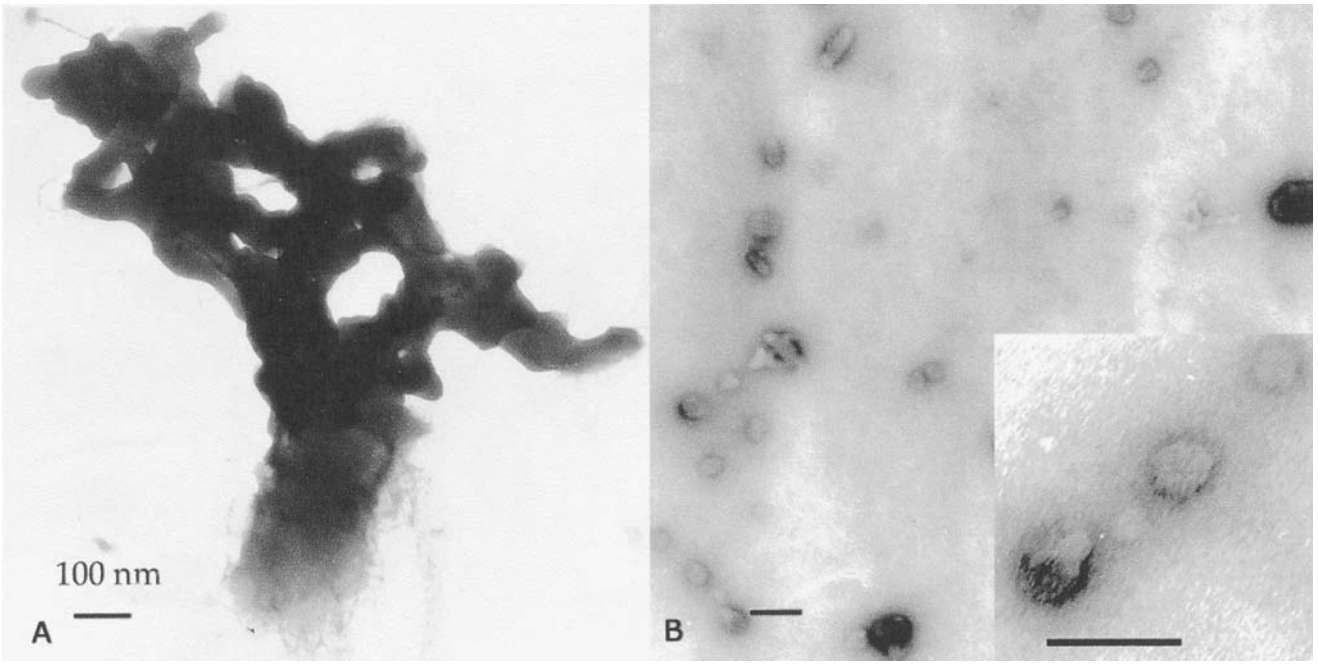

Figure 7 Electron micrographs of PEI 22-kDa–DNA complexes prepared in Nacl 0.15 M (A, N/P = 2) or in 5% (w/w) glucose (B, N/P = 5); bar represent 100 nm.

barrier in a manner similar to vectors based on the proteolytic C fragment of tetanus toxin (TTC peptide) (67).

B. Delivery to Respiratory Tract

Gene therapy to the lung could be exploited for both genetic and acquired diseases. However, like the brain, any therapeutic approach for the respiratory tract must take into account the heterogeneity of the cellular targets in the lung: epithelial cells, alveolar cells, vascular cells, and serous cells in the submucosal glands, etc. Two main routes can be used to introduce genes into pulmonary tissue: via the airways or through the circulation by systemic injection.

Both branched and linear PEIs have been tested with moderate success for delivering DNA by the tracheal route. Branched 25-kDa PEI gave higher levels of transgene expression than fractured polyamidoamine dendrimers especially when DNA–PEI complexes were prepared in water (68). Another group has tried nebulization of PEI–DNA complexes prepared in water (69). Their data show that PEI can protect DNA during nebulization and therefore give enhanced transgene expression. In a further analysis, they show that nebulization with 5% CO_2 improves transfection over that seen with air (70). Yet, in a recent study (71), PEI was shown to give exceptionally high (>1 ng luciferase/mg protein, ca. 1%–5% transfected tracheal cells) and more durable expression (>4 days) following slow instillation of the complexes into the mouse lung.

Following the demonstration that in 5% glucose DNA complexed with 22-KDa PEI provides small particles showing good stability and diffusibility in the brain, it was logical to test whether these particles would be stable enough to provide transfection of various organs following systemic injection. To this end, we analyzed transfection obtained in different tissues of the mouse after injection of varying quantities of DNA complexed with different N/P ratio through the tail vein. It was found that in all cases the lung showed the highest levels of transgene expression with both luciferase and the β-galactosidase genes (72,73). Anatomical analysis revealed pulmonary cells to be transfected and no signs of lesions or toxic effects. This suggested that the particles were crossing the pulmonary capillary barrier, although double labeling was not carried out. Later work showing the transfection of pulmonary cells was confirmed by double immunostaining with appropriate markers and passage of the pulmonary epithelium was shown to be both rapid and nontraumatic (74). Bragonzi et al. (56) used this model with GFP expressing vectors and tagged complexes to further the analysis. They also compared transfection performances of polyplexes formed with branched and linear PEIs to that of two lipoplexes. The most efficient vector for delivery by this route was linear 22 kDa. DOTAP gave reasonable levels of expression with naked DNA, GL-67A, and 25kDa PEI showing negligible transfection.

C. Tumor Targeting

Obviously, another major field of application for gene transfer technology is the treatment of tumors. Unmodified or modified PEI has been used with the aim of targeting lung metasta-

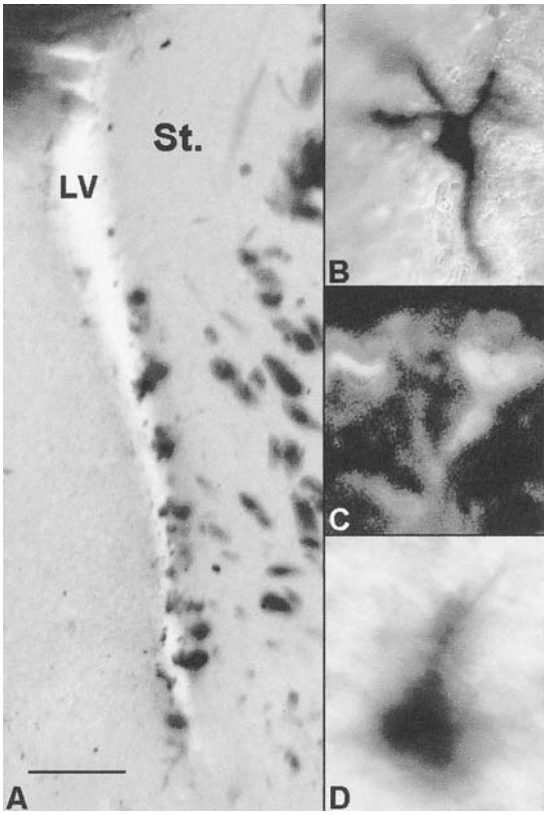

Figure 8 Privileged transfection of neuronal stem cells in adult mice with PEI. (A) In the adult CNS, intraventricular injection of PEI–DNA complexes results in transgene expression (β-gal positive blue cells), mainly on the striatal aspect of the lateral ventricles. (B–D) Identification of the transfected cells revealed a preferential transfection of the adult neural stem cells of the subventricular zone and their immediate progeny. (B) Positive cell with the typical morphology of astrocyte. (C) Confocal microscopy confirm GFP reporter gene expression in GFAP (marker of astrocytes)-positive cells in the subependymal layer. (D) Transfected cells (lacZ-positive cell) in this area also express Nestin, a marker of neural progenitors. Bar: (A) 200 μm; (B–D) 12 μm. St., striatum; LV, lateral ventricle. See the color insert for a color version of this figure.

ses or subcutaneous tumors (75). Various modes of delivery have been tested to this end: direct intratumoral of unmodified 22-kDa PEI (76), intravenous injection of transferrin-conjugated PEI (21) or even aerosolized PEI–DNA complexes to reach lung tumors (70). Interestingly, it is in this field of endeavor that multicomponent approach has yielded the best results. Kircheis et al. (43) shielded either 25-kDa or 22-kDa PEI–DNA complexes by covalently incorporating transferrin and used the complexes for systemic injection of mice bearing subcutaneous tumors. The presence of transferrin on the complexes provided a preferential transfection of the tumors rather than the lung.

VII. NUCLEAR MEMBRANE CROSSING

Whatever the delivery route used, probably the largest barriers to gene delivery are restricted cytoplasmic diffusion and the nuclear membrane (11,77). DNA particles entering fast dividing cells in culture are less hindered by these problems. Indeed, transfection of synchronized cells showed mitosis to be a key event for efficient transfection (78,79). It is therefore possible that, in general, DNA or DNA complexes that are unable to diffuse through the cytoplasm are dragged along with chromosomes and become incidentally sequestered in the nuclei of daughter cells during telophase. This would explain why most transfected cells appear as doublets (Fig. 9). Despite these observations, there are also quite well-documented cases of linear PEI-based transfection of nondividing cells both in vitro and in vivo. Indeed, in vitro transfection of resting (G1) HeLa cells with linear PEI has been shown to occur (46), albeit with 10-fold less efficiency than dividing ones (G_2/M). Lipids or branched PEI were much more dependent on cell division. Moreover, there are a number of studies indicating transfection of mature neurons in vitro and in vivo with linear PEI (62–65,80)

In vivo, even cancer cells can be considered as resting with respect to the lifetime of exogeneous DNA. A vectorial nuclear carrier should therefore be designed. Following the example of some DNA viruses, chemists attempt to divert the endogeneous nuclear import machinery by designing nuclear localization signal peptide (NLS)-bearing vectors (81–86). Unfortunately, cationic vector–DNA complexes are generally

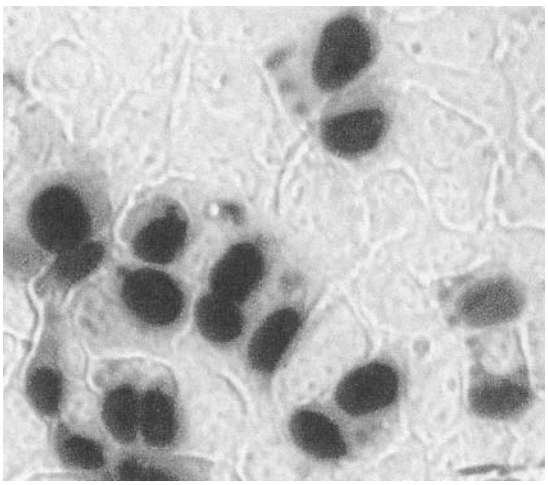

Figure 9 BNL. CI-2 hepatocytes transfected with nuclear LacZ/PEI complexes essentially appear as twins after 24 h. (Courtesy Dr P. Erbacher, Polyplus SAS.) See the color insert for a color version of this figure.

far too large to cross nuclear pores. Assuming the DNA–cationic vector complexes are (at least in part) disassembled in the cytoplasm, we have covalently bound a single NLS peptide to one end of a linear DNA fragment (Fig. 10). This hybrid construction was shown to enhance transgene expression up to 1000-fold in HeLa cells (87). Our working hypothesis was that the 3-nm-wide DNA-NLS molecules present in the cytoplasm would initially be translocated through a nuclear pore by the nuclear import machinery. As DNA would enter the nucleus, it would be condensed into a chromatinlike structure, which would provide the mechanism for threading the remaining wormlike molecule through the pore. Unfortunately, large enhancement factors were only observed for cell lines. Resting primary cells remained refractory, which may indicate that the large intracellular motions that occur during mitosis are required for intracellular trafficking of the DNA-NLS as well.

VIII. CONCLUSION

Following the initial findings over a decade ago that cationic lipids and polymers are able to carry genes into eukaryotic cells, much effort has been dedicated to improving transfection efficiency via structural modifications of the carrier. Exploiting structure–activity relations led to molecules capable of transfecting adherent cell lines in culture at a multiplicity of infection of ca. 10^6 gene copies per cell. In vivo gene delivery, the prerequisite to gene therapy, is still orders of magnitude less effective.

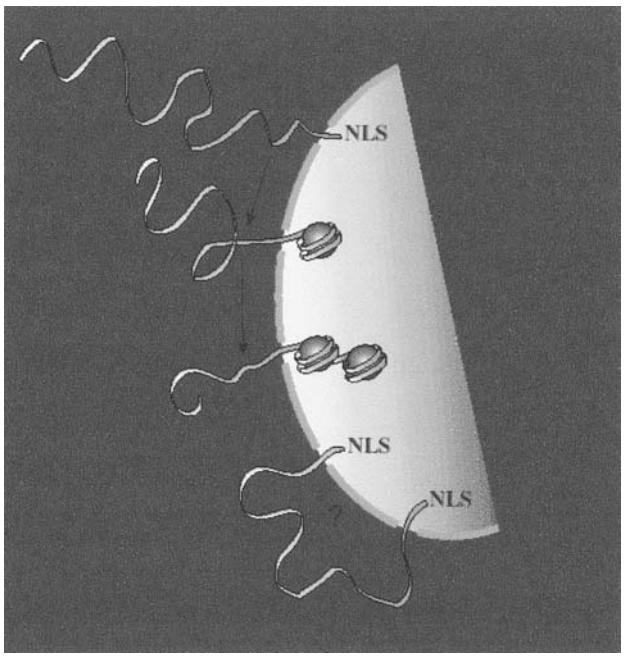

Figure 10 Nuclear pore crossing of a hybrid DNA-NLS peptide conjugate. See the color insert for a color version of this figure.

It does not seem reasonable anymore to search for ''the'' molecule able to carry DNA across the numerous barriers. Vectors of the future will be composed, much like viruses, of multifunctional supramolecular systems that self-assemble around DNA. Programmed intracellular disassembly may well be part of a successful story, too, especially for coated or reticulated particles. Each component of the vector is devoted to a particular function. As illustrated above, some functions, such as integrin-mediated cell entry or NLS-directed entry into the nucleus, mime viruses. Some other solutions, such as monomolecular genome condensation *via* detergent dimerization, or endosome release by the proton sponge effect, have obviously not been exploited by the natural cell invaders. The consequences of increased complexity of the vectors on their development as gene medicines (88) are difficult to evaluate at this stage. In any case, the puzzle still has to be assembled prior to ressemblance to an artificial virus (89): this is yet another story.

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13

Receptor-Targeted Polyplexes

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I. INTRODUCTION

Crucial requirements in gene therapy are a successful delivery of the therapeutic gene into the right target cells and a controlled expression in the transfected cell, at the appropriate levels and over the required period. The current gene delivery systems are not optimal in these aspects. During the last decade increased attention has been given to nonviral gene delivery vehicles, such as naked plasmid DNA, cationic liposome–DNA complexes (lipoplexes), polymer/DNA-based systems (polyplexes), and combinations thereof (1–2). These delivery systems are attractive because of their simplicity (they can be generated from few, defined components), and the flexibility in synthesis and assembly of the gene transfer complex. If desired, they can be designed protein-free/nonimmunogenic and can be very flexible regarding the size of DNA to be transported. However, their efficiency in gene delivery does not meet the requirements for most therapeutic applications.

Evolution has resulted in several mechanisms that enable the transport of molecules into cells that include not only simple diffusion, active transport, phagocytosis, receptor-mediated endocytosis, but also other mechanisms such as those used by various viruses to infect cells, bacterial conjugation, and natural transformation procedures seen in some microorganisms such as Helicobacter pylori (3). These natural pathways can be used in a new, artificial setting as means of a transfer route to deliver nucleic acid into cells. This article describes the nonviral delivery system, which exploits receptor-mediated endocytosis to target and transfer nucleic acids into cells.

The concept of receptor-mediated gene transfer draws its attention from the natural delivery mechanism based on receptor-mediated endocytosis (4,5). There are basically 2 mechanisms of endocytosis: (1) clathrin-dependent receptor-mediated endocytosis (coated pit endocytosis) (6), and (2) clathrin-independent endocytosis (7). Clathrin-dependent receptor-mediated endocytosis involves the binding of a ligand to a specific cell surface receptor, resulting in the clustering of the ligand–receptor complexes in clathrin-coated pits, invagination into the cell and budding off of the coated pits from the cell surface membrane to form intracellular coated vesicles, and maturation (uncoating, fusion of vesicles) into endosomes. Within these endosomes, ligands and receptors are each sorted to their appropriate (intra)cellular destination (e.g., lysosome, golgi apparatus, nucleus, or cell surface membrane). The clathrin-independent mechanisms resulting in uncoated pits include phagocytosis, pinocytosis, and potocytosis. Phagocytosis is a mechanism of internalizing large particles and microorganisms ($>0.5\ \mu m$). This mode of internalization is initiated by receptors on the phagocyte recognizing the particle either directly or indirectly, via opsonization of the particle. Internalization is primarily mediated via pseudopod action rather than pits (invaginations) on the cell surface. The engulfed particle, initially situated in the early phagosome, is eventually destroyed along the endocytosed pathway. Thus, cell capability to recognize particles via receptors and to form pseudopods seems to be a major characteristic mediating phagocytic internalization. Other forms of internalization that do not use clathrin, however, do seem to rely on pits (invaginations) on the cell surface. Such internalization systems include

macropinocytosis, pinocytosis, potocytosis, and transcytosis. Potocytosis and transcytosis may use caveolae as routes for internalization (5,8).

Many receptors contain motifs in their cytoplasmic domains that act as recognition sequences for initiating the process of enhanced intracellular uptake of macromolecules. With ligand interaction, the rate of receptor internalization is increased. Whether the intracellular pathways of the internalized molecule and biochemical consequences is the same between the different forms of endocytosis and ligands remains unclear (9–12).

To describe and elucidate the science of receptor-targeted polyplexes used in gene transfer, special attention is given to the many barriers that need to be overcome for gene transfer to be efficient. Factors such as DNA condensation, particle size of the DNA complex, route of administration, stability of the transferred gene in vivo, physical barriers that need to be overcome in order to reach target sites, and other in vivo confrontations, are discussed. The current concepts on binding of DNA complexes to the cell surface receptor and internalization and intracellular trafficking are reviewed, discussing also strategies for enhanced intracellular vesicular release, cytoplasmic trafficking, and nuclear targeting.

II. POLYPLEXES, CELLULAR TARGETING, AND RECEPTOR-MEDIATED ENDOCYTOSIS

A. DNA-condensing Carrier Molecules and Polyplexes

To transfer DNA safely and efficiently into the cell, it needs to be primarily stabilized. This is achieved by using carrier molecules capable of binding DNA. Such DNA-binding carrier molecules are also used not only to condense and compact the DNA into a size preferred by the target cell for being internalized, but also to neutralize the negative charges of the DNA, hence preventing repulsive forces between the DNA and the cell plasma membrane. In addition, carrier molecules may also protect the DNA from certain undesired affects of the physiological environment. DNA-binding carrier molecules should interact with the genetic material in a reversible, noncovalent, nondamaging manner. Cationic lipid-based systems, formulating DNA into ''lipoplexes,'' and cationic polymers, formulating DNA into ''polyplexes,'' have been used (1). For the majority of the cases, the cationic polymers—polylysine and polyethylenimine (PEI)—have been applied for binding and condensing DNA into polyplexes with sizes of 50 nm up to several hundred nm (13,14), which can be taken up by cells (15–19). A series of other DNA-binding polycations have also been used in polyplexes: polyarginine (20), protamine (13), or nonhistone nuclear proteins such as high-mobility group (HMG) proteins 1 (21), as well as nuclear proteins such as histones H1, H3, or H4 (22).

The cationic portion of the DNA complex can enhance binding to the cell (in addition to ligand-mediated receptor binding), and may also facilitate and mediate the transfer of the DNA to the cytoplasm, such as by disruption of the vesicular membranes. Thus, DNA condensation is essential for efficient transfer into cells; however, not any molecule that binds DNA results in an appropriate condensation.

B. Receptor Ligands for Targeting Specific Cells

Gene transfer vehicles have to fulfill several major delivery tasks: to transfer the therapeutic gene from the site of administration to the surface of the target cells, and to facilitate the internalization of the gene into the cells, trafficking to the nucleus. Incorporation of receptor ligands has been considered to support these tasks [i.e., targeting and enhanced cellular internalization of the transferred (therapeutic) gene, and in some cases, trafficking to and targeting to the nucleus, possibly including intranuclear trafficking]. Furthermore, nonspecific cellular internalization of a DNA–polycation complex via a net positive charge could result in cytotoxic side effects, such as disruption of cell membranes and aggregation of erythrocytes (23). Coupling ligands to the DNA-binding carrier (see Fig. 1) may overcome these obstacles by reducing positive surface charges of DNA complexes, preventing erythrocyte aggregation, and by enabling the complex to target specific cells, binding them strongly by ligand–receptor interactions, and thus minimizing interactions with nontarget cells (23).

Specific cell binding and enhanced cellular uptake can be mediated by 1 ligand, but could also be regarded as 2 separate processes because some ligands might target and bind a specific cell surface receptor, but not necessarily result in enhanced internalization, whereas other ligands have no cell-type specificity, but efficiently mediate uptake. The importance of these 2 points is very clear when working in vitro as well as in vivo. In vitro, targeting the gene of interest to specific cells is not a problem as such because the delivered gene gets in direct contact with the target cells only, and hence, need not seek out specific cells. However, it is necessary to enhance cellular uptake of the transferred gene. This can be achieved by using ligands as intracellular delivery-enhancing elements. For in vivo application, both cellular targeting and enhanced intracellular delivery of the polyplex are crucial for successful gene therapy.

Ligands, internalized via receptor-mediated endocytosis, represent a wide variety of macromolecules with varying physiological activities, including nutrient provision (e.g., low-density lipoprotein, transferrin); modified molecules from the circulation (e.g., ASGP, plasminogen activator inhibitor complexes); growth factors and hormones (e.g., insulin, VEGF, EGF), and some lysosomal enzymes. Some of these ligands may be coupled to DNA complexes (see Fig. 1), targeting them to specific cells. Ligands can be (1) proteins such as transferrin and asialoglycoproteins, and (2) small natural or synthetic molecules such as folic acid, peptides, or sugar derivatives. Examples of ligands that have been used as conjugates with polycationic carriers for targeted gene transfer are shown in Table 1 and references (9–10,13, and 24–87).

A general aspect to be considered in the selection of a ligand to be coupled to the carrier is that certain ligands are very spe-

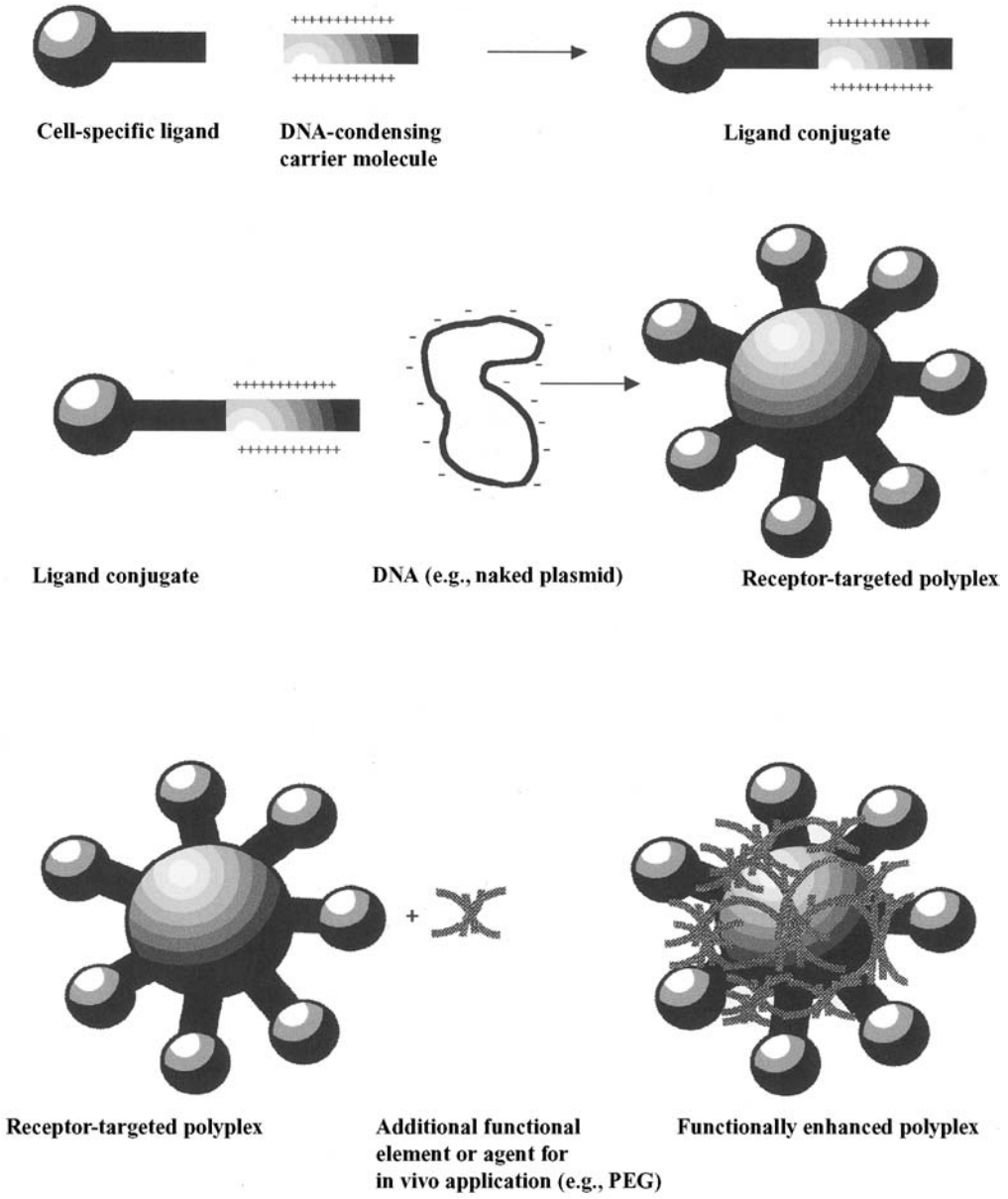

Figure 1 Assembly of DNA complexes (polyplexes).

cific in targeting certain cells or tissues in the body (e.g., asialoglycoproteins/hepatocytes), whereas others are not [e.g., transferrin/iron supply to many cell types]. Some ligands are internalized very efficiently (e.g., transferrin, anti-CD3 antibody bound to the T cell receptor-associated surface molecule CD3), whereas some others may be internalized either very slowly or not at all. Thus, the choice of ligand for efficient gene transfer is fundamental. Regarding this, one may also take advantage of the ligand binding and internalization enhancement as 2 separate processes, using 2 different ligands, 1 for targeting and the other for internalization. Hypothetically, 2 different ligands could also be used, where 1 ligand serves cell binding function only, whereas the other ligand enhances cellular internalization. The target cells need to contain cognate receptors, enabling the ligands to work in concert. Efficient internalization will only take place with cells containing both receptor types, enabling the 2 ligands to work in concert. Furthermore, the biology of specific cell types may also play an important role for efficient targeting. For example, cellular targeting ligand coupled to the DNA complex can be used as an element

Table 1 Ligands Used in Receptor-mediated Gene Transfer

Ligands	Refs.
Alpha2 macroglobulin	24,25
Anti-CD3	26,27
Anti-CD5	28
Anti-CD117	29
Anti-EGF	30
Anti-HER2	31
Anti-IgG	32,33
Antisecretory component Fab	34–36
Anti-Tn	37
Antithrombomodulin	38
Antibody ChCE7	39
Asialoglycoproteins	40–49
EGF	50–52
Fibroblast growth factor 2(FGF2)	9,53
Folate	54–56
Glycosylated synthetic ligands	57–69
IgG (FcR ligand)	32,70
Insulin	10,71
Invasin	72
Lectins	73–75
Malarial circumsporozoite protein	76
RGD-motif (integrin binding)	77
Steel factor (CD117 ligand)	78
Surfactant proteins A and B	79,80
Transferrin	13,81–87

to only target the corresponding cell, whereas internalization can be achieved via phagocytosis. Thus, cells containing the corresponding receptor will be targeted, but internalization will only take place if the cells contain competent phagocytic apparatus.

1. Ligand Conjugates

Cell-targeting ligands can be coupled to the DNA-binding elements (for examples, see Table 1), forming a conjugate capable of interacting with and condensing the DNA, and targeting it to specific cells (Fig. 1). The DNA-binding element (DNA carrier molecule) should be bound to the ligand, without its DNA binding, condensing, and protective functions being affected, and without affecting the cell targeting property of the ligand. Such a molecular conjugate containing both DNA-binding and cell targeting properties can be combined with DNA, forming the conjugate–DNA complex (''polyplex''). This polyplex (1) should contain the DNA in a highly condensed state, with the ligands positioned in a manner, free to interact with the target cell receptors.

Conjugates are commonly synthesized by covalently coupling the ligand to the DNA-binding polymer. Twenty years ago, reports were published describing the concept of exploiting natural endocytosis pathways of ligands for the delivery of DNA macromolecules. A method was published for the covalent coupling of DNA to protein ligands, such as alpha2-macroglobulin, formulating the concept of receptor-mediated endocytosis (24). Alternative approaches use compositions containing DNA associated with the ligand in noncovalent mode, using the ligand linked to liposomes (88). This concept was expanded, with approaches such as modifying proteins (transferrin and asialoglycoprotein) with positively charged N-acylurea groups that enable electrostatic binding to DNA, to generate DNA-binding ligands for receptor-mediated gene transfer (89). A chemically more defined approach involves conjugates of asialo-orosomucoid, and the polycation poly(L)lysine (40). Complexes of these DNA-binding conjugates with DNA plasmids encoding CAT marker genes or therapeutically relevant genes were shown to result in gene expression both in vitro in cultured HepG2 hepatoma cells, and in vivo in the liver of rats or rabbits (40–47). This was the beginning of the era ''receptor-mediated gene transfer'', where ligand–polycation conjugates were complexed with DNA, to condense, target, and transfer the gene into specific cells. Since then, many successful attempts have been performed in synthesizing ligand–conjugates, for example, conjugation of transferrin, folic acid, anti-CD3 antibody, to the DNA-binding elements polylysine, protamines, histones, intercalators, and complexing them with DNA for achieving targeted gene transfer (see Table I).

Taking transferrin as an example, conjugate synthesis involves the modification of transferrin and DNA-binding polycation with bifunctional reagents such as succinimidyl 3-(2-pyridyldithio) propionate (40,81). In the first steps, the activated (succinimidyl) esters can separately react with some amino groups of transferrin and the polycation. Subsequent steps result in disufide bonds (reducible) between the polycation and transferrin. Alternatively, a bifunctional reagent containing a maleimido group has been used (85), resulting in a (nonreducible) thioether linkage. Such an approach is not necessarily specific because the actual site of ligation between transferrin and the polycation is unknown. Conjugate synthesis can also be achieved in a more specific manner (e.g., via ligation through the transferrin carbohydrate moiety) (83). Transferrin contains 2 carbohydrate chains that are attached by N-glycosylation to Asn-413 and Asn-611. The glycan chains have a biantennary structure, composed of a core bearing 2 N-acetylneuraminyl-N-acetyllactosamine units. The glycosylation on the transferrin has no known influence on receptor binding or any other biological function (apart from the clearance of asialotransferin from the plasma). Thus, this site of the transferrin carbohydrate moieties is a good choice for attachment of polylysine and other nucleic acid-binding elements, without disturbing the cell receptor targeting characteristics of transferrin. To couple the transferrin to the amino groups of polylysine, the transferrin carbohydrate groups need to be activated. The terminal point of the transferrin carbohydrate chains consists of sialic acids. The 2 terminal exocyclic carbon atoms of the sialic acids can be selectively removed by periodate oxidation, resulting in the formation of aldehyde groups at the end of the carbohydrate chains.

The concept has been applied to other targeting ligands (Table I). Ideally, the chosen ligand must be recognized by specific cell surface receptors, bound with high affinity and internalized. Most research in this field has been performed by targeting the liver-specific asialoglycoprotein receptor and the ubiquitous transferrin receptor. Other possibilities, such as using antibodies to target specific cells, have been investigated successfully. Anti-CD3 antibody, coupled to carrier-DNA complex has been shown to be very efficient in targeting T cells via binding the CD3 T cell receptor complex (26). Malignant B cells have also been successfully targeted by aids of anti-idiotype antibodies (33). Others have achieved selective targeting of cells by using the ligand folic acid, to target cells that target the folic acid receptor (54–56). It has been demonstrated that the growth factor receptors HER-2 (31), EGF-R (30,51,50), and FGF2-R (9), also highly overexpressed in many human tumors, serve as good targets. Transferring genes into macrophages by mannose/fucose or galactose-specific membrane lectin is another example demonstrating that the receptor characteristics of cells can be exploited to target the cell via an appropriate ligand (57–69).

More recently, the DNA delivery activity of the cationic polymer PEI has been combined with the concept of receptor-mediated gene delivery by incorporating cell-binding ligands (transferrin, anti-CD3 antibody, lactose) by covalent linkage to PEI. Incorporation of cell-binding ligand results in an up to 1000-fold increased transfection efficiency (69,87). This activity was obtained with electroneutral particles, but depends on ligand–receptor interaction, resulting in enhanced cellular internalization. It is important to note that coupling of the ligand did not disturb the DNA condensing and transfer capability of PEI, which is a required aspect in formation of a DNA/conjugate polyplex.

In conclusion, enhancing cellular internalization by incorporating ligands to the carrier, not only enhances cellular internalization, but also avoids problems such as cytotoxicity of positively charged complexes, as well as undesired interactions with other cells and molecules, which would otherwise arise due to the need of a net positive charge of the DNA complex required for efficient (nonligand-mediated) transfection.

Alternative approaches include those that employ DNA-binding domains derived from transcription factors to attach a protein component to plasmid DNA (90). For example, plasmid DNA containing GAL4 recognition motifs can interact with GAL4-containing carrier molecules. Cell-specific ligands can be combined with the GAL4 domain to form a chimeric fusion protein to allow gene delivery to specific cells. For example, GAL4/invasin fusion protein has been shown to transfect target cells in an invasin receptor-dependent manner (91). However, for complex formation, and hence condensation, an additional condensing agent such as polylysine is required.

III. INTRACELLULAR TRAFFICKING AND BARRIERS

Once the cell has been targeted and the DNA has been internalized via receptor-mediated endocytosis, several intracellular barriers (Fig. 2) need to then be overcome for successful foreign gene expression to occur. To tackle the intracellular barriers, a polyplex needs to:

- Escape from the endosome
- Survive the cytoplasmic environment
- Traffick the cytoplasmic environment targeting the nucleus with subsequent nuclear entry
- Be disassembled in the nucleus so it can be recognized by the cell's transcription machinery and be expressed under control in the target tissue population only

Overcoming the intracellular barriers may be achieved via certain DNA carrier molecules (92) and cell targeting ligands, although exploiting other factors such as additional endosomal releasing mechanisms and nuclear localization signals could strongly improve the capacity of a polyplex to overcome intracellular barriers.

A. Endosomal Escape

The first major intracellular barrier that greatly impairs the efficiency of gene transfer is the entrapment and degradation of the DNA complex within intracellular vesicles after budding off of the coated pits from the plasma membrane (Fig. 2). Entrapment and degradation can be regarded as 2 separate barriers because overcoming vesicular degradation would only result in the accumulation of the transferred gene in the vesicles, while still limiting further transport into the nucleus. Thus, after cellular internalization, the transferred gene needs to overcome enzymatic degradation during vesicular fusion into lysosomes, and once this is done, it needs to then be released from the vesicles to traffick the cytoplasm and target the nucleus.

Several strategies have been developed to ensure the protection/release of DNA complexes from intracellular vesicles. The strategies involve incorporation or linkage of vesicular destructive elements to polyplexes, which perturb the integrity of vesicle membranes, allowing the luminal contents to spill into the cytoplasm in a nondamaged manner. The fundamental aspect here is to perturb the vesicular membrane, without damaging the DNA complex and other cellular membranes. Thus, the endosome-releasing mechanisms need to become active within the membraneous vesicle, without rupturing the cellular membraneous structures and organelles.

Application of endosome (vesicular) releasing agents to the transfection process has shown to augment gene transfer. Lysosomotropic agents, glycerol, virus particles, membrane disruptive proteins and peptides, and photosynthesizing compounds possess properties that can reorganize and disrupt vesicular membranes, promoting the release of polyplexes (see below).

1. Lysosomotropic Agents

Lysosomotropic agents are weak-base amines that can inhibit lysosomal function specifically (93,94). Examples of such agents are ammonium chloride and the weakly basic alkylamines, such as methylamine, propylamine, chloroquine, procaine, and spermidine. These agents are termed lysosomotropic

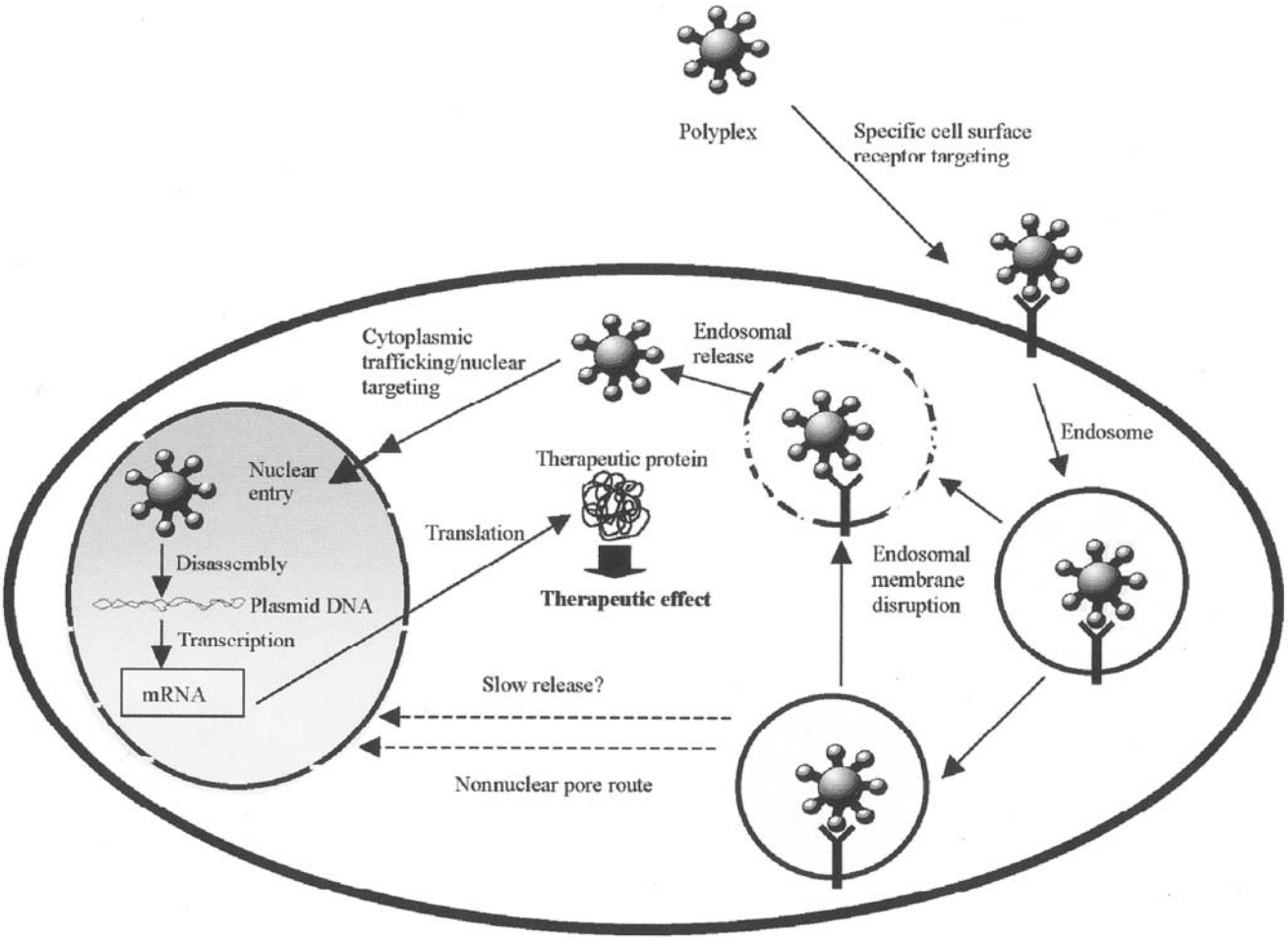

Figure 2 Intracellular release and trafficking of polyplexes.

because they accumulate in the endosomes/lysosomes. This accumulation is partly due to the initially low lysosomal pH, and partly because of the continuous pumping of protons into the endosome/lysosome.

In some cell lines, such as erythroid cell line K562, gene expression is strongly enhanced by adding chloroquine to the transfection medium. This enhancement is primarily due to the prevention of intravesicular (lysosomal) degradation, followed by vesicular membrane disruption triggered by osmotic effects (see below). Chloroquine is also the most commonly used lysosomotropic agent in gene transfer experiments (82). It is believed that chloroquine accumulates in the endosomal/lysosomal compartment, acting osmotically, vacuolarizing and eventually disrupting the vesicle. In this case, the transferred gene is protected and released from intracellular vesicles, bafilomycin or monensin, 2 other agents that also prevent endosomal/lysosomal acidification but do not accumulate or enhance transfection. Regarding chloroquine, its effectiveness in enhancing gene transfer is also dependent on cell type, which ensures vesicular accumulation of the lysosomotropic agent. For example, K562 cells, in comparison to other living cells, have a defect in their vesicular pump system, determining the accumulation of chloroquine in endocytic vesicles (95). The use of chloroquine is limited due to cytotoxic properties.

2. Glycerol

Glycerol is a trihydric sugar alcohol, being the alcoholic component of fats. Several reports describe the interaction of glycerol with cellular membranes (96,97). Incubation of DNA–polylysine complexes in the presence of glycerol has resulted in a substantially enhanced transfection efficiency in primary fibroblasts and some cell lines. Regarding this, glycerol probably acts in intracellular vesicles after DNA complex internalization, rather than at the cellular membrane. The presence of glycerol alone is not sufficient for efficient gene transfer. For glycerol to have its maximum effect, other factors such as DNA complex net positive charge and the type of DNA-binding carrier plays a major role. Thus, a combined action of glycerol and

polylysine on vesicular membrane seems to be responsible for enhancing gene transfer (97). It should, however, be noted that many cell types do not show the strong enhancement by glycerol.

3. Virus Particles

Adenovirus particles are capable of inducing endosomal lysis during the process of adenoviral infection. Upon acidification of the endosome, the capsid proteins (e.g., the penton proteins) undergo conformational changes to an active form, capable of disrupting the endosomal membrane causing release of the contents into the cytoplasm. This observation potentiated the exploration of using adenovirus (viruses that enter cells via receptor-mediated endocytosis) as a means of overcoming vesicular entrapment during gene transfer. Adenovirus has been successfully incorporated in DNA complexes, enhancing transfection efficiency 100- to 1000-fold (48,49,73,84,98–105). Incorporation may be achieved by adding adenovirus freely, where endomolysis action is in trans, or by directly linking the virus to the DNA complex. Various methods of linkage have been applied as an alternative method for ensuring cointernalization of the adenovirus, which is not always the case when the virus is added in trans. The majority of these methods have been investigated with DNA complexes with polylysine as the DNA-binding element. There are several methods of covalently coupling polylysine to the exterior of the adenovirus. Approaches, including an enzymatic transglutaminase method or chemical coupling methods, have been applied successfully. Alternative approaches are those involving noncovalent coupling methods, such as an immunologic linkage strategy, with an anticapsid monoclonal antibody effecting the linkage between the adenovirus and polylysine DNA-binding moiety (antibody bridge), ionic interactions, or biotin-streptavidin bridge. Such complexes consisting of DNA, adenovirus-polylysine, and ligand-polylysine are referred to as ''ternary complex.''

Only the membrane destabilizing function of the adenovirus capsid is required, thus the viral genome can be inactivated with methoxypsoralen plus irradiation, retending endosome disruption properties (99,102). One problem with the inclusion of adenovirus is that the transferred gene may also enter cells via the adenovirus receptor, thus compromising ligand-specific gene transfer. Strategies to inhibit uptake via adenovirus receptor include coupling of polylysine to periodate oxidized adenovirus. This treatment modifies the adenovirus fiber, which is necessary for virus attachment. An alternative approach is to target an antibody against the adenovirus fiber. Ablating binding to adenovirus receptor does not interfere with subsequent endosomolyic activity. In addition to human adenovirus, CELO (chicken adenoviral strain) has also been successfully linked to DNA complexes, enhancing receptor-mediated gene transfer, although not as efficient as adenovirus (73). However, a major drawback in using viruses is their inflammatory response of cells to virus entry per se as well as their immunogenicity.

Because many other viruses also enter cells via the endocytic pathways, they may be used as gene transfer enhancers, provided that they display an endosomolytic function. The use of human rhinoviruses (picornaviruses), which are RNA viruses, has been described and investigated for releasing vesicular-entrapped DNA complexes into the cytoplasm (106). One major drawback of using rhinoviruses is the toxicity to human cells due to host protein synthesis machinery shut off. One way to circumvent the viral-induced drawbacks is to use only the endosomolytic portion of the virus, or synthetic derivatives thereof.

4. Membrane Disruptive Proteins and Peptides

For many biological processes such as entry of viruses and bacterial toxins into cells to be exercised, cellular membrane barriers need to be by passed, and this is usually achieved by membrane reorganization processes. The membrane reorganization process is the result of specific actions of certain membrane-disruptive elements (peptides and proteins). In most cases, the membrane active locate in such elements are peptide domains with amphipathic sequences. Under appropriate conditions the amphipathic sequences can interact with lipid membranes, perturbing them. This characteristic can be specifically used to influence gene transfer; membrane-active peptides can be derived from viral peptide sequences such as the N-terminus of influenza virus hemagglutinin subunit HA-2 or the N-terminus of rhinovirus VP-1, or they may be designed synthetically from the derived peptides by molecular modeling (e.g., GALA, KALA, EGLA, or JTS1) (106–110).

Viruses that enter the cell via the receptor-mediated endocytosis pathway have evolved specific mechanisms that ensure the release of their genome from the intracellular vesicles into the cytoplasm. The mechanisms leading to vesicular release are associated with viral proteins that specifically perturb membranes, either in a disruptive or fusogenic manner. The membrane-disruptive peptides are tools of membrane-free viruses (e.g., adenovirus), whereas the membrane-fusion peptides belong to enveloped viruses (e.g., influenza virus). These fusogenic or endosomolytic protein domains are activated in a pH-dependent manner, and this characteristic can be used to enhance gene transfer (106–110) in a similar manner as whole virus particles, as mentioned above. The virus-derived peptides can be incorporated into polylysine–DNA complexes either by covalently linking to polylysine or via biotinylation of the elements, which allows binding to streptavidinylated polylysine. Simple noncovalent ionic interactions between positively charged polylysine–DNA complex and the negatively charged residues of membrane-destabilizing element is another possibility to achieve linkage. The linkage procedures should be also applicable to other DNA-binding elements. The membrane-destabilizing peptides have shown to enhance receptor-mediated gene transfer up to 1000-fold in cell cultures.

Other larger viral proteins have also been investigated for enhancing transfection by endosomal release. Adenovirus penton proteins have been attached to DNA via a synthetic oligolysine-extended penton-binding adaptor peptide. This complex transfers the DNA into the cell via adenovirus receptor and results in intracellular release (111).

One of the characteristics involved in virulence activity of micro-organism is the formation of substances (toxins, enzymes, and other proteins) that cause damage to the host (112,113). Some of the virulence factors act specifically on cell

membranes. This action can be direct, for example, action of streptolysin O, resulting in cell lysis, or indirect, specifically acting on intracellular vesicular membranes, resulting in the release of entrapped virulence factors, having had entered the cell via an endocytic pathway. Various bacterial lysing proteins (cytolysins) and other toxins have been used as devices to enhance gene transfer. For example, streptolysin O and staphylococcal alpha toxin have been used standardwise as intracellular delivery reagents. Perfringolysin O has also been used to enhance delivery of DNA into cultured cells (114). For this purpose, the biotinylated protein was bound to DNA–polylysine complexes by a streptavidin bridge. Another well-studied exotoxin capable of enhancing gene transfer is the diphtheria toxin. A recombinant transmembrane domain of this toxin has been coupled to polylysine with subsequent incorporation into DNA–asialoorosomucoid-polylysine complexes (115). Also, the bee venom component melittin has been applied for enhancing polyplex-mediated transfection (116).

5. Slow Release from Vesicular Reservoirs

Partial hepatectomy or microtubular disruption by colchicine treatment has shown to prolong gene expression after intravenous injection of DNA–asialoorosomucoid-polylysine complexes (46,47). This prolonged expression is due to the continuous existence and survival of plasmid DNA in cells of treated cells. This survival is due to protection of the DNA complex from the lysosomal or cytoplasmic environment, as a result of DNA complex persistence in the endosomal compartment, and the prolonged gene expression is due to a constant slow supply of plasmid DNA to the nucleus. The slow release is not a direct endosomolytic action. Therefore, how the DNA complex reaches the nucleus in this case remains unclear.

6. Photosensitizing Compounds

A novel technology, named photochemical transfection or photochemical internalization, has been recently developed and is primarily based on photosensitizing compounds, such as tetra(4-sulfonatophenyl)porphine or aluminium phthalocyanine, that localize in the membranes of endosomes and lysosomes (117,118). These become activated upon illumination and induce the formation of reactive oxygen species, destroying endosomal membrane structures releasing endocytosed DNA into the cell cytoplasm. This technology is being currently investigated as a new strategy for cancer therapy (119).

7. Cationic Carriers

In contrast to DNA-binding elements such as polylysine, there are other polycations that possess specific properties enabling them to combine DNA binding and condensing activity with membrane-perturbing capacity, thus, not requiring the presence of endomolytic agents for enhancing transfection. The membrane-perturbing activity of such DNA-binding elements may be associated with their ionic state, or conformational flexibility, resulting in membrane-specific interactions.

PEI and polyamidoamine polymers (''dendrimer'') are efficient transfection agents per se (15–17). Most of these agents possess buffering capacity below physiological pH. This buffering capacity is due to residues of these agents not being protonated at physiological pH, making them efficient ''proton sponges.'' Upon acidification in the intracellular vesicle, the further protonation of the polymers triggers chloride influx, resulting in osmotic swelling (endosome swelling), and thus destabilization (rupture) of the intracellular vesicle membrane, resulting in the escape of the DNA complex. Hence, gene transfer is enhanced in the sense that DNA is free to travel to the nucleus.

Histidinylated polylysine, optionally in combination with zinc ions, was also found to have enhanced transfection property, presumably mediated by a related vesicular escape mechanism (120). Moreover, there are designed cationic peptide carriers that can bind nucleic acids and permeabilize lipid bilayers at the same time. One example is the cationic amphiphilic peptide KALA mentioned above (110).

B. Cytoplasmic Trafficking, Nuclear Targeting, and Entry

Upon receptor-mediated endocytosis and release from the endosome, polyplexes need to then overcome cytoplasmic degradation and traffick to and enter the nucleus. These tasks are only partially fulfilled by certain DNA carrier molecules and cell targeting ligands. Other elements such as nuclear localization signals can be incorporated into polyplexes improving nuclear targeting.

The nucleus is separated from the cytoplasm by the nuclear envelope, which consists of 2 chemically distinct membranes, the inner and outer membrane, with the perinuclear cisterna space in-between. The outer membrane is continuous to the endoplasmic reticulum. Nuclear pore complexes (NPCs) span the nuclear envelope, and transport of macromolecules from the cytoplasm to the nucleus occurs through these complexes. Regarding nuclear import, several major processes need to be distinguished: (1) the steps leading to the import process; that is, signals guiding the protein transport to the nuclear membrane, including cytoplasmic recognition of the import protein by transport factors and nuclear pore targeting; (2) the actual molecular mechanism of translocation of the protein from the cytoplasmic side of NPC to the nuclear side of NPC into the nucleus; the nuclear pores being the site of translocation; (3) release of the import protein into the nucleus; and (4) recycling of transport factors.

There are several distinct nuclear import signals that guide the import of proteins into the nucleus. These signals are part of the primary sequence of the protein destined to be targeted into the nucleus. The best characterized ones are the SV40 LTA ''classical'' nuclear localization signal (NLS) and M9 (an import signal of hnRNP AI protein) import signals.

It has been suggested that shuttling of import receptors may be a major process in nuclear transport of proteins. In this hypothesis, the import receptor binds the protein in the cytoplasm. The molecule is then carried through the NPC into the nucleus, where it is released from the transport receptor. The receptor returns to the cytoplasm, ready for transporting the next molecule into the nucleus. In this model, the binding of the transport

receptor to the molecule may be regulated by the different environments of the nucleus and cytoplasm.

Four major transport factors are required for the NLS-dependent protein import: (1 and 2) importins alpha and beta (karyopherin alpha and beta), (3) GTPase Ran/TC4, and (4) nuclear transport factor 2 (NTF2) (p10). These factors interplay for successful transport of proteins into the nucleus. Proteins (with NLS signal) with sizes up to 25 nm interact in the cytoplasm with soluble NLS receptor (importins alpha and beta). The karyopherin beta 1 (''importin beta'') mediates docking to nucleoporins, the components of the nuclear pore, located at cytoplasmic face and nucleoplasmic face of the NPC. Nucleopore cytoplasmic filaments may also be involved in the nuclear targeting process. GTPase Ran and p10 (NTF2) are required to translocate the docked NLS peptide into the nucleus. The influenza virus nucleoprotein particle is one example that is taken up by this pathway.

The M9 domain of hnRNP A1 contains 38 amino acid residues (M9 import signal) sufficient for import purposes into the nucleus. It bears no sequence similarity to classical NLS. Nuclear import of ribonucleoprotein A1 is mediated by another distinct import pathway, by binding to karyopherin beta 2 (''transportin''—import receptor of the M9 pathway). It also requires Ran.

It is very likely that, in addition to the classical NLS and the M9-type import, more pathways into the nucleus exist. This may be of an advantage in the sense of regulating import of distinct classes of molecules separately (121,122).

The nuclear envelope represents a major barrier for polyplex-mediated gene transfer. In the majority of cell types, transport of DNA into the cell nucleus is inefficient. For example, less than 1% of NIH 3T3 fibroblasts have shown to express β-galactosidase after cytoplasmic injection of reporter gene. However, β-galactosidase has been efficiently expressed when injected into the cytoplasm of primary rat muscle cells. Although how DNA complexes find their way to the nucleus is not fully understood, recent findings suggest that DNA compaction (92), certain physiological ligand pathways such as bFGF-targeted complexes (53), or specific DNA sequences, as demonstrated by intact protein free SV40 DNA (123), may contribute to cytoplasmic trafficking and nuclear targeting of the polyplex.

In dividing cells, DNA may passively enter the nucleus during mitosis when the nuclear membrane is broken down (124). However, many cell types are nondividing with the nuclear membrane staying intact, thus, the transferred gene needs to enter the nucleus differently than in dividing cells. In this case, nuclear entry and trafficking may represent a major interintracellular barrier for successful in vivo gene transfer. When DNA is injected into the muscle cell far from the nuclei, expression decreases. This may be the result of cytoplasmic sequestration, preventing nuclear accumulation of DNA. The movement of DNA toward the nucleus may be inhibited by its binding to cytoplasmic elements, and/or entrapment within the cytoskeletal mesh (125). However, polymers such as PEI and PLL have been suggested to promote gene delivery from the cytoplasm to the nucleus, possibly attributed to intrinsic nuclear targeting activity and protective mechanisms against cytosol nucleases (126,127).

Once the polyplex has trafficked to the nucleus, it then needs to enter through the nuclear membrane into the nucleus. Although rupture of the nuclear envelope (as occurs during cell division) is not a necessity for some PEI polyplexes to penetrate the nucleus (92,128), transfection efficiency of polyplexes in general is critically dependent on cell division (129). High transfection efficacy has been observed when polyplexes are added to cells in late S or G2 phase, and low transfection when polyplexes are added in G1 phase. Thus, transfecting nondividing cells in vivo may be considered as an additional barrier in vivo in terms of nuclear entry of polyplexes.

Thus, understanding the mechanisms of nuclear entry is critical and will be a large step toward designing gene delivery systems, more efficient in transferring the DNA into the cell nucleus.

Expression of transferred DNA has been inhibited by wheat germ agglutinin (WGA), suggesting that DNA may enter the nucleus by the WGA-sensitive process common to large karyophilic proteins and RNA (125) (WGA blocks the NPC machinery because of the presence of N-acetylglucosamin residues on nucleoporins). However, recently it has been shown that nuclear localization of DNA does not require the addition of cytoplasmic protein factors necessary for protein import (130). Nevertheless, DNA entry appears to be regulated by NPC. The NPC accommodates both passive diffusion and active transport. Molecules smaller than 20 nm in diameter passively diffuse through NPC into and out of the nucleus. Larger macromolecules require active transport for nuclear entry. The exact mechanism by which exogeneous DNA passes through the NPC has not yet been determined, although it may be similar to the transport of proteins, larger than 15 Kda, actively into the nucleus.

Certain viruses such as hepatitis B virus also use the active nuclear import mechanism. Viral core particles containing synthesized DNA bind to the nuclear pore complex; the viral polymerase, which is covalently linked to the viral DNA, acts as NLS. Core particles (35 nm) exceed the maximal diameter of the nuclear pores, thus requiring disassembly before the viral genome is internalized.

The efficient transport of DNA complex into the nucleus is an active process that probably requires a nuclear localization signal. Several NLSs have been identified in proteins with a nuclear fate, the sequences being very basic, with >50% of their amino acids being lysine residues [e.g., Phe-Lys-Lys-Lys-Arg-Lys-Val, directing the nuclear import of SV-40 large T antigen, or the NLS (Lys-Lys-Lys-Tyr-Lys-Leu-Lys) within HIV-1 matrix protein]. Substitution of 1 of the lysine residues results in total failure of nuclear import. Thus, it is possible that DNA-binding elements rich in lysine (e.g., polylysine) may play a role in the nuclear import of DNA complexes. Regarding this, injection of DNA–polylysine mixtures into the cytoplasm of mouse ES cells has lead to transgenic animals with about 50% efficiency (compared with intranuclear injection of naked DNA). In contrast, injection of naked DNA into the cytoplasm does not lead to transgenesis. To make the DNA import process more efficient, it may be necessary to direct the transport to the nu-

cleus by incorporating NLSs into the DNA complex. This is strongly supported by recent findings (131) that show that incorporation of a single SV40 LTA NLS into a DNA plasmid molecule can dramatically enhance transfection efficiency.

To sum it up, it would be desirable to engineer DNA complexes with specific nuclear targeting and translocating elements, enabling the DNA complex to be (1) recognized in the cytoplasm by nuclear import receptors (by interacting with transport motifs either directly on the DNA sequence or on a protein from the DNA complex), (2) targeted to the nuclear pore complex, (3) translocated efficiently through the nuclear pore, and (4) released at the nucleoplasmic face of the nuclear pore complex into the appropriate nuclear compartment for subsequent disassembly and expression.

The cell nucleus is crowded, containing large amounts of DNA, RNA, and protein. In addition, nuclear processes such as replication, transcription, translation, and DNA repair processes are constantly active in specific compartments, resulting in a nuclear jam (132,133). Within all the mass of cellular DNA, RNA, and proteins and the nuclear processes, the DNA complex needs to become (either disassembled or reorganized in a suitable fashion) exposed, enabling the nuclear expression machinery to recognize and express it. Premature disassembly and DNA release from the carrier molecule in the cytoplasm may prevent efficient transfer to the nucleus, hence obligerate expression. Polyplexes most likely disassemble in the nucleus, which is a characteristic desirable for successful gene transfer and expression (92).

Furthermore, the anatomical location (intranuclear depot) of polyplexes within the nucleus may be crucial for efficient expression (53). For example, bFGF is believed to be retained in the nucleus within discrete storage depots. Similar distributions have been reported for bFGF-targeted polyplexes with a relatively poor level of specific gene expression, suggesting difficult availability of the DNA for transcription (53). Indeed, intranuclear trafficking of transcription factors contributes to transcriptional control, and thus, has implications for biological control (134,135); hence, it may determine intranuclear trafficking/localization if used as carrier molecules (90,91).

Whether incorporation of intranuclear targeting elements in polyplexes affects nuclear localization and hence, expression, remains to be considered for investigation. Thus, both efficient uncoating and intranuclear trafficking is another challenge to be tackled.

C. Persistence of Gene Expression

Several factors endanger the persistence of transferred DNA within the nucleus: (1) degradation by intranuclear nucleases; (2) DNA loss, mainly during cell division, although DNA may also be rapidly lost even when cells are not dividing; (3) loss of transfected cell because of apoptotic, inflammatory, or immune response; (4) silencing of the introduced gene by transcriptional shut-off; and (5) possibly inefficient intranuclear trafficking.

Protection of DNA may be achieved via the proper ratio of DNA–DNA-binding carrier (e.g., polylysine). Saturating the binding capacity of the DNA backbone with the DNA-binding moiety may avoid the access of DNA to nucleases and increase the stability of the DNA in the nucleus (nuclear retention, resulting in prolonged expression).

DNA loss other than degradative loss can be prevented by including specific sequences to the transferred DNA that ensure either integration of the DNA into host chromosome, or extrachromosomal replication of the transferred DNA with equal segregation to daughter cells. These persistence-ensuring sequences can be derived from certain viruses or from chromosomes.

Retroviruses and adeno-associated virus (AAV) stably insert their genome into host genome. The integration mechanisms have been characterized [retrovirus: LTR sequences, integrase protein; AAV: inverted terminal repeat (ITR) sequences, rep protein], and may be exploited by incorporation of the corresponding nucleic acid and protein elements into a viruslike particle (DNA complex). During its lysogenic cycle, AAV integrates into a specific site, denoted AAVS1, on human chromosome 19 (136). This property has been used to achieve site-specific integration of plasmid DNA in 293 cells. When the AAV-encoded recombinase rep is supplied in cis or trans, plasmids containing the AAV ITRs are integrated at AAVS1. In this way, DNA can be directed to a specific region of a human chromosome, and thus may avoid the problem of random insertional mutations created by integrative vectors such as retroviral vectors.

Other viruses such as herpes virus (e.g., Epstein Barr Virus, EBV) can persist in infected cells without integrating their genome into the host. This persistence is partially due to replicative property of viral DNA via *cis*-acting origin of replication, which is activated by the *trans*-acting gene product of the viral EBNA-1, and additional nuclear retention mechanisms. The viral-persistence mechanisms can be used in designing extrachromosomal-replicating DNA constructs (episomal vectors) by integrating the appropriate sequence elements, recognizable in mammalian cells, into the DNA construct to be transferred (e.g., EBV Ori P, EBNA-1). DNA constructs containing these sequences have the ability to replicate once per cell cycle with nuclear retention without interfering with the host chromosomes. An alternative to using viral origin of replication, human genomic sequences may be used to mediate DNA construct replication (137).

Other origins of replication characterized include those of bovine papilloma virus or SV40 (138). However, these replication origins also require viral proteins for activation, which may have oncogenic or toxic properties. The viral replication origins are species specific and may also replicate more than once per cell cycle, resulting in mutations in the transferred gene.

The next challenge is the generation of artificial chromosomes for maintenance of large genomic sequences. The basic DNA sequence requirements for human chromosome function are believed to be similar to those identified in yeast, which include a centromere, telomeres, and origins of replication. These elements have been used to construct yeast artificial chromosomes (YACs) (139), and their transfer has been demonstrated by spheroblast fusion. Researchers are now trying to construct better artificial chromosomes (140). It is important here to de-

fine the minimal sequence requirements for functional mammalian chromosomal elements.

Human artificial chromosomes (HACs) may serve as valuable DNA constructs, containing the requirements for achieving gene expression persistence. An opening door toward this goal is the recent generation of mitotically and cytogenetically stable artificial chromosome derived from transfecting human HT 1080 cells with alpha satellite plus telomere DNA and genomic carrier DNA, forming *de novo* microchromosomes with functioning centromeres (141). The only sequence that has been shown to form *de novo* centromeres after transfection is alphoid DNA with telomere DNA and genomic carrier DNA. However, centromeric function of human chromosome is not always associated with alphoid repeats. There are other nonalphoid genomic regions characterized by neocentromic activity (sometimes, a centromere can appear at a new position in a chromosome by a process called ''centromere activation''). Transfection experiments with DNA present at the neocentromere should demonstrate whether this DNA can also form centromeres *de novo*.

With advancement, HACs could be used to introduce therapeutic genes into cells. To use HACs for human gene therapy, efficient methods for delivery will be required. The receptor-mediated gene transfer system is a very promising choice capable of targeting and transferring any large DNA construct into cells. Bacterial artificial chromosomes have already been delivered into mammalian cells using psoralen-inactivated adenovirus/PEI carrier (103).

Persistence of the transferred gene is required, but not necessarily sufficient. It does not necessarily mean that gene expression is going to be efficient and long-lasting. A failure of gene therapy in clinical trials may not always be due to gene delivery, but rather due to novel confrontations at the expression level. Specific expression cassettes determine the efficiency of expression after the gene has been transferred and maintained in the nucleus. The major elements of the expression cassette are those that ensure strong, controllable, (switchable), and cell specific (tissue restricted) expression of the therapeutic gene. Previous studies using viral promoters (e.g., cytomegalovirus promoter/enhancer) have observed a transcriptional shut-off (''silencing'') of the introduced expression cassettes. This can be avoided by the use of natural, cell-specific promoter/enhancer sequences. These are also attractive due to the opportunity of transcriptional targeting, as a further filter for specificity (142). Some examples are steroid-inducible promoters, tumor-specific promoters, muscle-specific promoters, hypoxia response elements, hepatitis B virus-derived promoters for liver-specific expression, and multidrug-resistant gene promoter (143). Here lies the attractiveness of nonviral receptor-targeted polyplexes in gene therapy, for the reason that any gene construct regardless of the size may be transferred.

IV. EXTRACELLULAR BARRIERS FOR GENE DELIVERY

For in vivo applications, the development of polyplexes is not only confronted with intracellular barriers, but also with extracellular barriers, which are those prior to reaching the target cells. A schematic diagram of the potential intracellular and extracellular fate of a polyplex is shown in (Fig. 3). Strategies have to be developed in designing polyplexes that enable them to survive blood and other biological fluids, and to escape extracellular physical barriers, in order to reach the target cells. The specific strategies must take into account the physicochemical properties such as size, shape and flexibility, overall charge, charge density, and nonelectrostatic interactions at the surface of the polyplex. Besides considering these physical properties, one could imagine to also use active endogenous cellular transport mechanisms, such as transcytosis (12,144).

In vitro gene delivery and ex vivo gene therapy approaches are predominantly concerned with gene delivery barriers presented by the cell itself. In vivo gene therapy must also be concerned with extracellular barriers, where the route of administration of the therapeutic gene also plays a major role (145). In systemic administration, where the vector is introduced in the body intravenously, the circulatory pathway and environment, as well as various nontarget cells and organs, encountered by the polyplex are major obstacles, determining the fate of the transferred DNA [see, for example, (146)]. However, local administration methods, such as direct injection of the vectors into the target region, are not confronted with the circulation problem, but nevertheless are still confronted with barriers such as extracellular matrix or inflammatory and immune responses.

A. Physical Restrictions of Transfection Particles

Size seems to be a general critical factor for drug targeting (147). Because of size restriction, (several) hundred nm large particles cannot penetrate endothelial and epithelial barriers (148), or extravasate from the vascular to the interstitial space. Particle size is also an important factor when considering organ clearance and intraorgan distribution. For example, particles that are too large to pass through the vascular endothelium to the liver parenchym are engulfed and degraded by liver Kupffer cells (i.e., phagocytic cells residing next to the vascular epithelial cells).

DNA is a negatively charged, flexible molecule, both characteristics that hinder the DNA from being transported efficiently into and across cells, which in this case represent the physical barriers. To overcome these physical drawbacks, the DNA molecule needs to be compacted and the negative charges minimized for efficient gene transfer. These requirements can be achieved by taking advantage of molecules capable of binding and condensing DNA (149). The structure of condensed DNA complexes has been analyzed in several reports, such as (13,150–152,14). DNA–polycation complexes have been characterized by electron microscopy and atomic force microscopy (shape, size), laser light scattering (size), electrophoretic mobility (reflects charge and size of complexes), zeta potential measurements (charge), circular dichroism (conformation of DNA), or centrifugation techniques (molecular weight and condensation). The results give some insight on how to possibly generate DNA complexes sufficiently small to traverse the en-

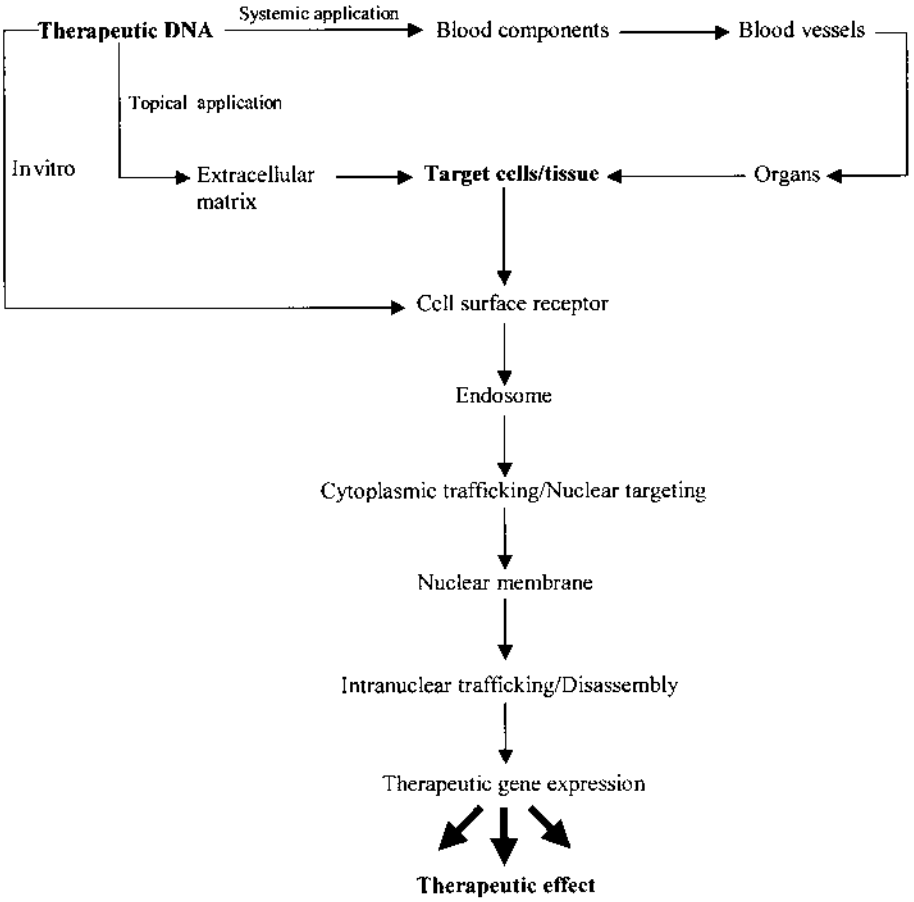

Figure 3 Extracellular and intracellular pathways for gene delivery. In vivo, systemic or local application; in vitro, cultured cells.

dothelial layer, possibly through fenestrations or vesicular transport systems, such as transcytosis (Fig. 4). In addition, the compact DNA complexes are more stable against enzymatic or mechanical degradation, which may take place during DNA transport processes to the target cells/tissue.

However, preparation of effective DNA complex molecules for in vivo delivery of DNA remains a major hurdle. The extent of DNA condensation depends on a number of variables, including the ratio of positively charged DNA-binding element (''cationic carrier'') to negatively charged DNA, the size and modification of the DNA-binding element, size, sequence, and state of the DNA, and also the procedure of complex formation (29), strongly influencing the in vitro and in vivo gene transfer efficiency. The net charge of the DNA–cationic carrier complex affects its solubility. Complexes with either an excess of DNA or positively charged carrier are stabilized in solution by the negative or positive charges. At molar charge ratios [see (1): positive charges of carrier to negative charges of DNA phosphates] close to 1 (i.e., electroneutrality), hydrophobic domains of DNA-binding elements such as polylysine are considered to be responsible for low solubility in water. This may lead to aggregation and precipitation of complexes.

Methods of formulation still have to be improved in generating homogenous and stable complexes capable of overcoming the physical barriers. DNA–polylysine-conjugate complexes have been prepared in several ways. Wu and Wu (40) mixed the compounds at high salt concentration, where electrostatic binding is strongly reduced. Slow reduction of the salt concentration by dialysis into physiological buffer results in a thermodynamically controlled complex formation. Charge ratios of polylysine/DNA smaller than 1 and enhanced hydrophilicity due to the conjugated asialoglycoprotein are presumably essential for the solubility of the complex.

Wagner et al. (13) described a different approach using transferrin-polylysine–DNA complexes. Flash mixing of dilute compounds in physiological phosphate-free buffer results in formation of kinetically controlled complexes. Charge ratios of polylysine/DNA from smaller than 1/2 to larger than 2/1 have been applied. At ratios of electroneutrality or higher, donutlike and rodlike particles of 80 to 120 nm in diameter are formed.

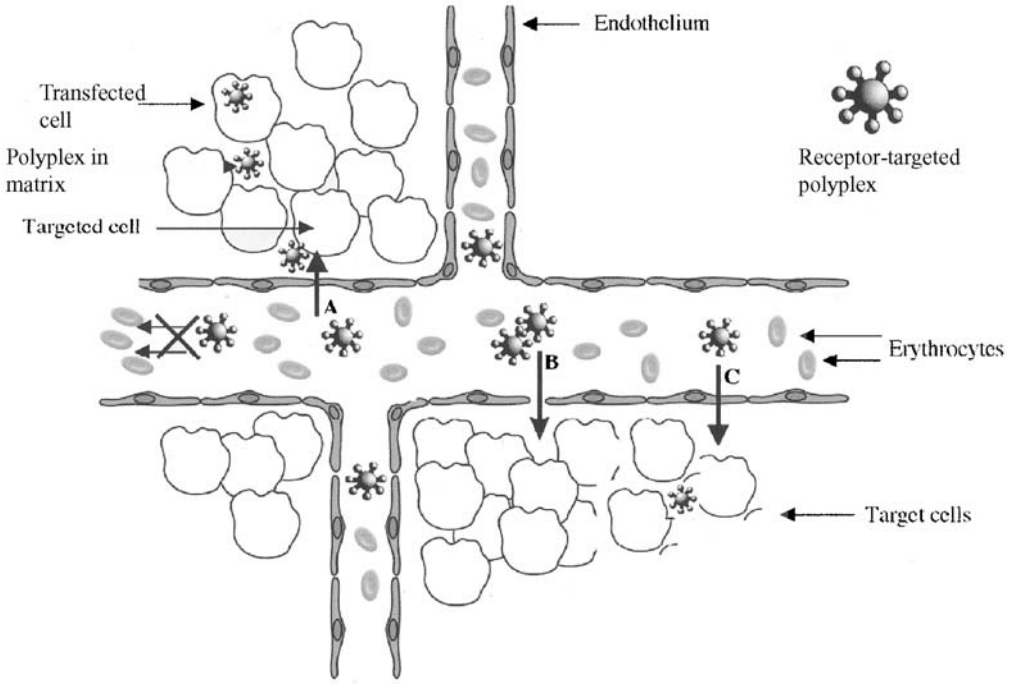

Figure 4 Vascular barrier for gene delivery. See the color insert for a color version of this figure.

Complexes containing transferrin-conjugated polylysine have increased solubility compared with the use of unmodified polylysine.

Interestingly, donuts of similar sizes are formed, independent from whether small or large (up to 48 kbp) and DNA is used in the complex formation. Using a standard expression plasmid of approximately 5 kbp, obviously several DNA molecules are incorporated into 1 particle. In an attempt to generate unimolecular DNA complexes, Perales and colleagues (35,60) added polylysine conjugates slowly, in several small portions, to a vortexing solution of DNA in approx. 0.5 to 0.9 M sodium chloride until a charge ratio of polylysine/DNA of approximately 0.7 is reached. The slow addition of polylysine has been reported to generate monomeric DNA complexes, with sizes of approximately 15 to 30 nm. These complexes aggregate immediately; aggregation is reverted by subsequent addition of salt. The promising findings have been reported to be applicable for in vivo gene transfer applications (35,60).

Recent reports on the size of DNA complexes with PEI or transferrin-PEI describe the strong influence of parameters such as DNA concentration and charge ratio, and also ionic strength of solution, or serum content of culture medium. Mixing DNA–PEI complexes at N/P (PEI nitrogen: DNA phosphate) molar ratios below 6 in 150 mM saline results in rapid aggregation; aggregation can be avoided by complex formation at low ionic strength (25 mM aqueous buffer), generating particles with an average diameter of approximately 40 to 50 nm (14). Incorporation of hydrophilic polyethylene glycol residues into polyplexes also was found to stabilize small polyplexes and prevent their aggregation (153).

B. Undesired Interactions with Plasma, Degradative Enzymes, Matrix, and Nontarget Tissue

Polyplexes, when administered in vivo, are surrounded by a variety of compounds present in blood plasma. Salts, lipids, carbohydrates, proteins, or enzymes contribute to changes in the physicochemical properties of the polyplex. Some of these factors (''opsonins'') may coat the polyplex, causing aggregation, dissociation, or degradation of the DNA complex. This may influence the composition of the complex as well as the bioavailibility. Thus, the DNA complexes, even when reaching the target cells/tissue, may no longer exhibit the physical properties necessary for efficient transfer into cells.

Previous studies have demonstrated the inactivation of polylysine-based DNA complexes by blood components (154). One of the factors was identified as the complement system (155). More recently, the interaction of DNA–PEI complexes with plasma was analyzed on the biochemical level. Upon incubation of the DNA complexes with human plasma, specific proteins (IgM, fibrinogen, fibronectin, and complement C3) bind to the complexes (153). By coating the DNA–PEI complexes with polyethylenglycol (PEG) through covalent coupling to PEI, plasma protein binding was found to be strongly reduced (153).

Another problem encountered in the bloodstream is degradative enzymes. There are nucleases in the bloodstream that degrade extracellular DNA (such as that generated by degradation of invading microorganisms or dead host cells). Cationic DNA-binding elements may serve some protection (45).

Other undesired interactions are the ones with the extracellular matrix and nontarget cells/tissue. There is a complex network of proteins and proteoglycans, termed extracellular matrix that fill the intercellular space (156,157). The matrix helps bind the cells in tissues together and also provides a lattice through which cells can move. Once the DNA complex has traveled across the vascular barrier into the interstitial space, it has to avoid interactions with the extracellular matrix in order to reach and bind the target cells/tissue. Extracellular matrices in animals are composed of different combinations of collagens, proteoglycans, hyaluronic acid, fibronectin, and other glycoproteins. These components could serve as specific barriers by binding the DNA complexes. For example, hyaluronic acid binds cations very effectively. With this in mind, DNA condensed by polycations, resulting in a net-positive charge, could also interact with the extracellular matrix, binding, dissociating, or aggregating the DNA complex. Thus, proper formulations, in preparing the DNA complexes, will be necessary, to avoid such interactions. Optimizing the DNA complexes bearing an overall low-charge ratio close to neutrality is a possibility to avoid such interactions. The problem is that a net-positive charge has been found desirable for interaction with the cellular plasma membrane and entry into the target cell, whereas such positive charge might favor entrapment of the DNA complexes by negatively charged extracellular matrix components.

Interaction with nontarget cells/tissue is another hurdle, complicated to combat. In the previous sections, cellular internalization mechanisms using receptor-specific ligands were presented as a solution to overcome this problem. Ligands may target cells very efficiently, but it has to be kept in mind that they do not inhibit unspecific interactions with nontarget cells, which could result in cellular binding and internalization via any additional process. The unspecific interactions with nontarget cells may be due to factors such as particle size, charge, and in vivo protein coating of the DNA complexes. For example, interaction with nontarget cells may take place due to an excess positive charge of the complex. It has been shown in cell culture that minor changes in the DNA/polylysine-conjugate ratio of the complex, resulting in a positively charged DNA complex, may convert a ligand-specific transfer into a completely unspecific process (29). Ideally, the DNA complex should be masked in a fashion that only allows ligand–receptor interactions with the target cell, and no other interactions with nontarget cells.

C. Inflammatory and Immunological Responses

As a result of introducing foreign molecules into the body, individual immune cells are stimulated to produce antibodies, a process termed humoral immunity. In addition to this humoral response, specific T cells may also be activated (cellular immunity). These 2 processes are the specific immune response. There is, however, also the nonspecific immune response, including phagocytosis, inflammation, and other nonspecific host-resistance mechanisms such as the complement system (158). These nonspecific mechanisms develop immediately against virtually any foreign molecule, even those the host has never encountered. Thus, the nonspecific immune response is a major extracellular barrier for the DNA complex, which in this case is the foreign molecule. The ultimate goal is to formulate and construct polyplexes in a manner, that avoid eliciting any immune response.

Inflammatory response is a major problem for any gene delivery system (159) because it may take place independent from the route of administration and results in a greater access of phagocytes to the foreign molecules, for example, due to an increased capillary permeability caused by retraction of the endothelial cells. During an inflammatory response, leucocytes, particularly neutrophil polymorphs and to a lesser extent macrophages, migrate out of the capillaries into the surrounding tissue. At the site of inflammation, the phagocytes recognize the foreign molecules via receptors on their surface, which allow them to attach nonspecifically and phagocytose-foreign molecules. Attachment is greatly enhanced and specified upon opsonization of foreign molecules, such as by the C3b component of complement. Both neutrophils and macrophages have receptors that specifically bind to C3b, allowing them to recognize their target.

A variety of macromolecules, such as proteins, lipoproteins, some nucleic acids, and many polysaccharides, can act as immunogens under appropriate conditions. Positively charged DNA complexes have the ability to activate the complement system (155). A number of synthetic cationic molecules, frequently used in gene delivery, and their complexes with DNA have recently been examined for their complement-activating properties. Complement activation by polylysine is strongly dependent on chain length and on the charge ratio. Longer chains and greater surface charge density are strong activators of the complement system. The positive charges on the DNA complex are accessible to the complement protein C3b. Opsonization of such particles by C3b leads to the initiation of a cascade of events, presumably resulting in the clearance of DNA complexes by the retinoculoendothelial system. Coating of the positive charges of the DNA complexes with other macromolecules may inhibit interactions with components of complement, hence decreasing complement activation and clearance of the complexes from the blood circulation (160). It has already been demonstrated that modification of the surface of liposomes reduces interaction with blood components (161–163), stabilizes DNA–liposome complexes (164). Coating of polycation–DNA complexes by polyethyleneglycol (PEG) also reduces interaction of DNA complexes with blood components. (153,155).

V. POTENTIAL CLINICAL APPLICATIONS

Although gene transfer via receptor-targeted polyplexes are mainly successful in delivering DNA efficiently in cell culture,

several reports have recently demonstrated the capability of receptor-targeted polyplexes to deliver and transfer DNA to target cells systemically in vivo. This advancement has been enabled by using the various methods to overcome the intra- and extracellular barriers described in the previous sections, but also to a large extent by shielding (or coat) the positive charge of polyplexes, reducing their positive surface charge (zeta potential), hence preventing common interactions with extracellular barriers, such as nonspecific interactions with plasma components or erythrocytes. Shielding methods include those with PEG (153), poly-N-(2-hydroxypropyl)methacrylamide (pHPMA) (165), poloxamer (166), or recently ligand density (23).

A. Receptor-mediated Gene Delivery In Vitro (Ex Vivo)

One approach for performing somatic gene therapy is the ex vivo strategy. In this approach, gene transfer is performed in cell culture (in vitro) and the resulting transfected is transplanted into the organism. Application of receptor-mediated gene transfer to transfect endothelial cells (101,167), fibroblasts, B cells, vessels, and primary tumor cells has potentiated several ex vivo approaches. For example, the clotting factor VIII, which is deficient in hemophilia A, can be produced by transfected primary fibroblasts at levels more than 10-fold higher than those generated by retroviral vectors, enabling factor VIII expressing fibroblast implants for in situ expression of this protein (168).

The highly efficient delivery in vitro has resulted in the development of other ex vivo approaches. For example, treatment for malignant melanoma has been designed by application of gene-modified cancer cell vaccines. DNA complexes are used to deliver immunostimulators genes (e.g., interleukin-2) into melanoma cells in vitro. After irradiation (to block tumor cell growth), the transfected cells are applied in vivo to trigger an antitumor immune response. This treatment has been translated into a medical protocol and is being evaluated in clinical trials (169,170).

B. Receptor-mediated Gene Delivery In Vivo

The first encouraging results for in vivo gene transfer were obtained by targeting, via intravenous injection, the liver asialoglycoprotein receptor using asialoorosomucoid covalently linked to poly(L)lysine carrying the CAT marker gene (42–44,46,47). DNA expression proved to be liver specific. Other organs such as kidney, spleen, and lungs did not produce detectable quantities of CAT activity. Gene expression persistence was improved by applying partial hepatectomy, a procedure for stimulation of hepatic regeneration, and DNA synthesis, beginning 12 h after surgery, resulting in CAT activity up to 11 weeks postsurgery.

Related approaches have been used to target the liver, with different size DNA complexes. Unimolecular, about 12-nm small galactosylated DNA–polylysine complexes encoding human factor IX have been shown to target the hepatic asialoglycoprotein receptor, with up to 140 days of detectable protein in serum of transfected rats (60). This result was achieved without partial hepatectomy. In a related approach, the polymeric immunoglobulin receptor has been targeted by 25-nm DNA complexes bearing antigen-binding fragment of an antibody enabling gene transfer to rat pneumocytes following intravenous administration (35,36). It appears that the procedure in preparing DNA complexes may at least partially determine the success of in vivo gene transfer approaches, by influencing extracellular barriers.

Initially, polyplexes based on polylysine did not demonstrate efficient gene transfer in vivo. However, addition of endosomal disruptive agents, such as adenoviruses (84), to such polyplexes resulted in efficient gene expression in airway epithelium after intratracheal application (171) and in tumors after intratumoral application (172,173). Shielding of the polyplexes and the mode of administration, such as with micropump, have further advanced polyplexes to overcome such barriers as extracellular matrix and interstitial pressure (174), respectively. Local injection of DNA complexes directly into subcutaneously growing tumors produced significant reporter gene expression, with DNA–transferrin-PEI complexes or adenovirus-linked DNA–transferrin-polylysine complexes being 10- to 100-fold more efficient than naked DNA (173).

Recently, the lung has been successfully targeted via systemic tail vein injection of mice by 22-kDa linear PEI/DNA polyplex (175,176). High levels of gene expression were observed in the lungs but lower expression in other major organs, including heart, spleen, kidney, and liver. This observation indicated PEI to be suitable for transfection of the lungs. However, in contrast to the high transfection efficacy observed with PEI in the lungs, systemic gene delivery with PEI/DNA polyplexes to target lung tumors in mice did not result in such an efficient transgene expression (174). Thus, such polyplexes can be classified as nontarget complexes where several parameters may account for the pronounced systemic gene delivery to normal lungs but not to tumors. These include nonspecific interactions of positively charged polyplexes with negatively charged surface structures in the lungs, as well as aggregation of erythrocytes, resulting in lung embolism and severe toxicity (23).

C. Tumor-targeted Polyplexes

The promising results discussed above suggest that receptor-targeted polyplexes might be used to deliver genes systemically in vivo to target tumor-specific receptors or receptors that are differentially expressed on tumors (172).

Intravenous application of standard transferrin-PEI–DNA complexes through the tail vein into tumor-bearing mice (subcutaneous tumors) resulted in gene expression in the tail and lung; there was no expression in the tumor, but serious toxicity (153,173). However, surface shielding the transferrin-linked polyplexes with PEG through covalent coupling to PEI, complexes were stabilized in size, did not bind plasma proteins (153) and did not result in erythrocyte aggregation. These PEGylated PEI polyplexes, when injected into tail vein of syngeneic

mice (see Fig. 5) were far less toxic; gene expression in the tumor was up to 100-fold higher than in other organs (153,173). Similar results were obtained with other transferrin polyplexes with electroneutral surface: shielded optimized adenovirus-linked transferrin-polylysine polyplexes (173) or transferrin-PEI polyplexes with a higher content of transferrin as shielding agent (23).

Because subcutaneous tumors are not directly supplied by main blood vessels, delivery is dependent on peripheral blood supply. Thus, the polyplexes probably target such distant tumors in a combinatorial passive (i.e., overcoming extracellular barriers, EPR = enhanced permeability and retention effect in tumors) and active targeting mechanisms (i.e., specific receptor-targeted endocytosis).

Another recent report demonstrated that intravenous injection of PEGylated EGF-containing DNA–PEI complexes results in a highly specific expression in human hepatocellular carcinoma (HCC) tumors. Following intravenous injection into human HCC xenograft-bearing severe combined immunodeficiency mice, luciferase reporter gene expression was predominantly found in the tumor, with levels up to 2 logs higher than in the liver (52).

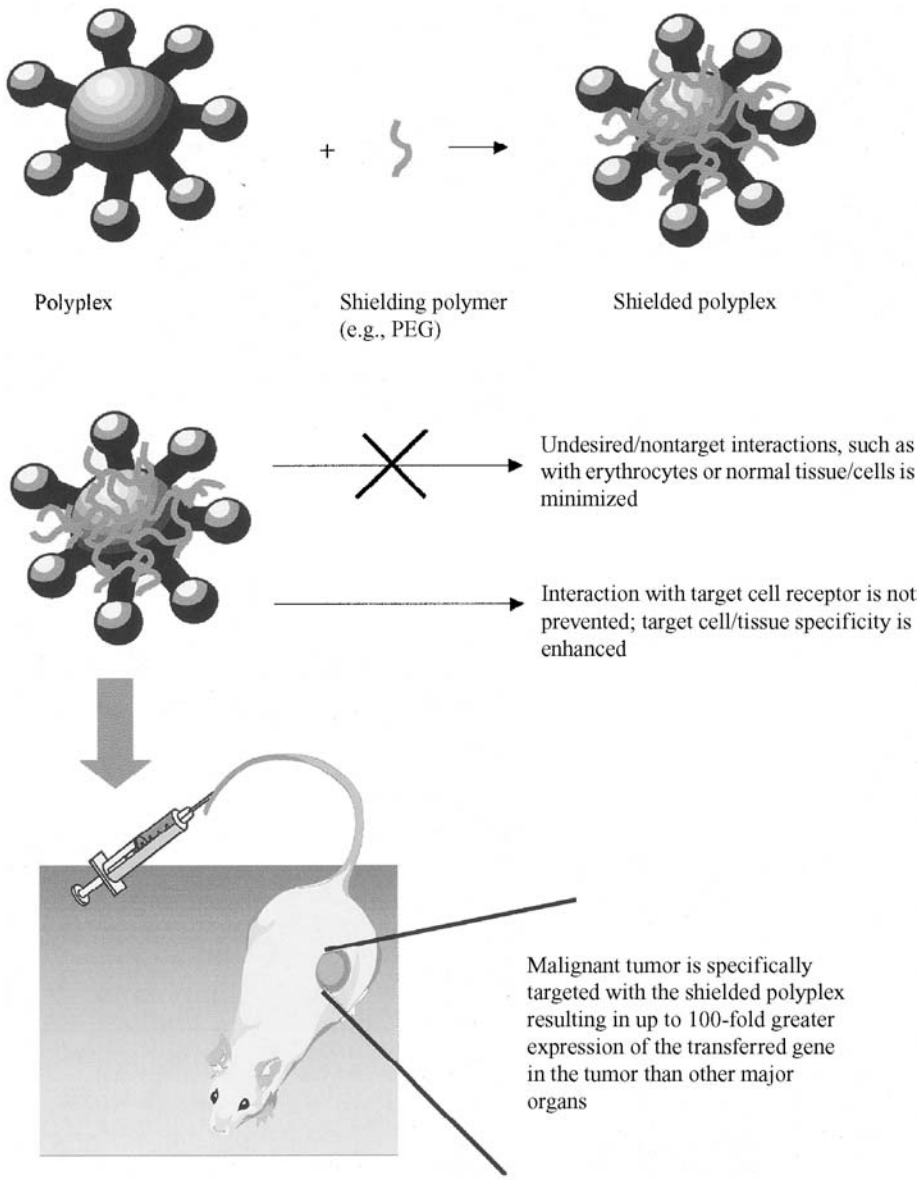

Figure 5 Tumor-targeted gene transfer. See the color insert for a color version of this figure.

Such shielded tumor-targeting polyplexes appear to also have interesting characteristics from a therapeutic perspective. Expression of tumor necrosis factor-α (TNF-α) as the therapeutic gene after systemic application of transferrin- or transferrin-PEG-shielded PEI polyplexes resulted in hemorrhagic necrosis in targeted distant tumors without TNF-α-related systemic toxicity (177,178). These first results are encouraging, and further optimization of tumor-targeting polyplexes should result in the development of potential therapies for human patients.

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14

Electrokinetic Enhancement of Plasmid Delivery In Vivo

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I. INTRODUCTION

An ideal delivery system for in vivo gene transfer should be simple and safe, and provide prolonged expression of transgenes at therapeutic levels. A variety of nonviral delivery systems can be used for gene transfer in different clinical settings (1). Plasmid constructs are attractive candidates for direct injection into organs and tissues because plasmids are well-defined entities that are biochemically stable and have been used successfully for many years (2,3). In a few cases, the relatively low expression levels achieved after simple plasmid injection are sufficient to reach physiologically acceptable levels of bioactivity of secreted peptides (4,5).

One of the most striking recent advances for plasmid delivery in vivo has been that of electropermeabilization, also described as either electroporation or electrokinetic enhancement. This physical process exposes the target tissue to a brief electric field pulse that induces temporary and reversible breakdown of the cell membrane. During the period of membrane destabilization, a variety of molecules, including drugs and plasmids, may gain intracellular access. A scheme of the electroporation process is depicted in Fig. 1. This method of molecule delivery, which is simple, efficient, and reproducible, has become valuable for basic research, with great potential for gene transfer and DNA vaccination (6). Electroporation has been used very successfully to transfect tumor cells after injection of plasmid (7,8) or to deliver the antitumor drug bleomycin to cutaneous and subcutaneous tumors in humans (9,10). Electroporation has been extensively used in mice (11–13), rats (14,15), dogs (16), and pigs (17,18) to deliver therapeutic genes that encode for a variety of hormones, cytokines, enzymes, or antigens. The numerous tissues and organs that have been targeted include liver, skin, eye, testis, cardiac muscle and smooth muscle, tumors at different locations, and skeletal muscle.

II. MECHANISTIC STUDIES OF ELECTROPORATION

The degree of permeabilization of the muscle cells is dependent on the electric field intensity, length of pulses, shape and type of electrodes, and cell size.

A. Electric Field Intensity

The influence of electrode configuration on the electric field distribution has been shown by measuring ^{51}Cr-EDTA uptake in vivo (19,20). These studies indicated two uptake phases in which the membrane permeabilization increases, followed by irreversible membrane damage. Both plate electrodes (18,21) or a pair of wire electrodes (22) have been shown to be effective. The calculated electric field distribution was more homogenous for plate electrodes than for needle electrodes. Also, the needle diameter determines 3 parameters: in rabbit liver, the decrease of the electric field intensity near the electrodes is steeper with smaller electrode diameter; the area covered with a given electric field is smaller with smaller electrodes (23); and there is a more homogenous electric field with larger electrode diameter.

Recently, square-wave electric pulses of different field strength and duration have been evaluated. Data for high-voltage pulses of short duration or low-voltage pulses of long duration are found in Table 1. Despite the large variation in conditions used for the target tissue or organ, the expression achieved in all these cases represents efficacious levels of gene products, some of which are within the range that is necessary to treat human diseases.

Theoretical and practical data also suggest that the cell size in the region perpendicular to the electric field plays a crucial role in determining the permeabilization parameters (24). The larger the ''functional'' size of the cell, the lower is the field strength necessary (25). For example, tissues containing cells

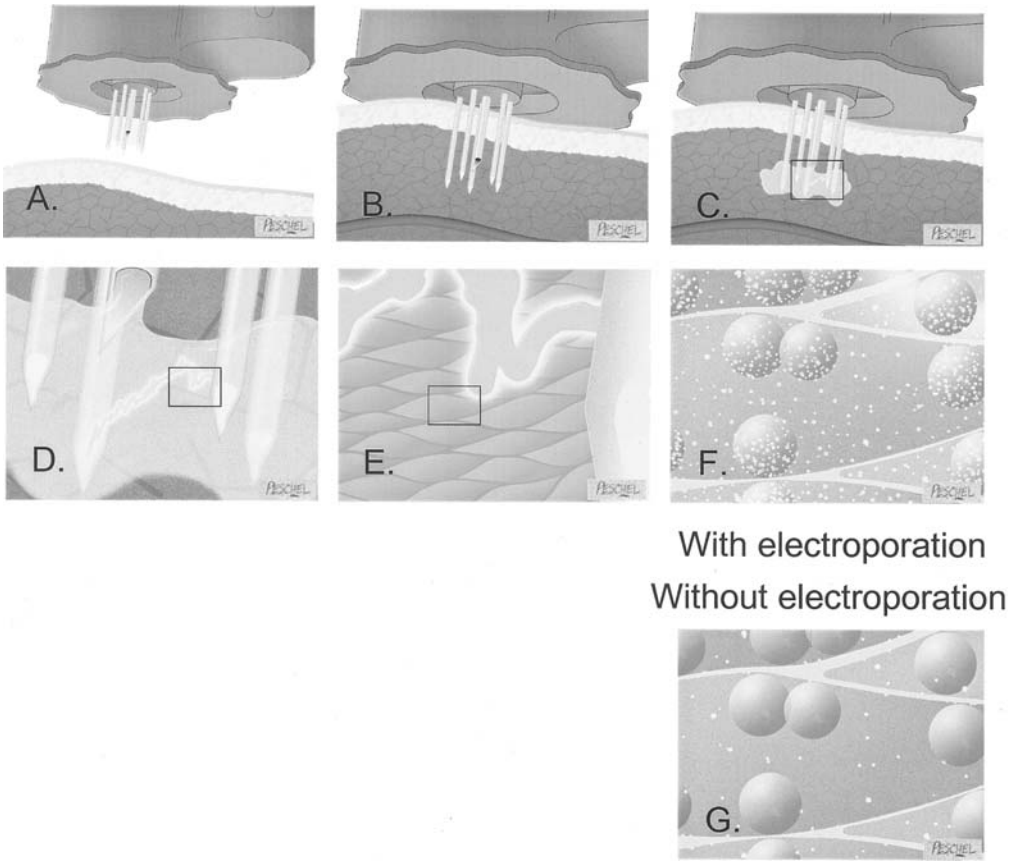

Figure 1 Schematic depiction of the electroporation process in the skeletal muscle using penetrating electrodes. (A) Electroporation system is placed next to the target muscle; (B) the injection needle and the electrodes are inserted into the muscle; (C) plasmid is injected into the target muscle; (D) at a selected time interval after plasmid injection, typically 2 min, a square-wave electric pulse is generated; (E) effect of the electric field at the muscle fiber level; (F) plasmid injection followed by electroporation increases uptake by 10- to 1000-fold into the target muscle; notice the muscle fiber nuclei that contain numerous copies of the injected plasmid; and (G) direct plasmid injection into the skeletal muscle, without electroporation results in a low plasmid copy number uptaken and expressed by the muscle fiber. See the color insert for a color version of this figure.

that communicate through tight gap junctions amplify transmembrane potential changes. Thus, the skeletal muscle can be electroporated at lower field intensities (26) with decreased tissue damage, compared with other tissues.

In studies of the electric field-mediated enhancement of gene and drug delivery, different types of electrodes have been used. These include clamp or caliper, tweezers, paddles, and needle arrays. In large mammals such as pigs, dogs, or humans, the increased resistance of the skin, the thickness of the subcutaneous fat tissue, and the concern for tissue damage that occurs with the proportionally increased intensity of the electric field makes use of some percutaneous electrodes inefficient and impractical. Thus, the electrode design must be adapted to the application, organ, and animal species. For cutaneous and subcutaneous tumors in animals and humans, calipers or needles (27–29) are used. For liver, tweezers, or occasionally needles (23,30–32); for skin, calipers, meander electrode, plate-and-fork electrodes, or small needles (29,33,34); and for the skeletal muscle, calipers or needles (17,18,35) are used.

B. Association with Carrier Molecules

Despite the recent advances in the technology of plasmid transfer into tissues, additional improvements in electroporation techniques and plasmid formulations are needed. For example, the entire electroporation procedure in theory can be completed without causing permanent damage to the cell. However, under some conditions, electroporation procedures inflict fatal stress on some skeletal muscle cells (16,36) and

Table 1 Examples of Square-wave Pulses Electric Field Intensity and Pulse Length

	Species	Tissue	Condition (Ref.)
High voltage	Rat	Skin	10 × 1000 V/cm, 0.1 ms (71)
Short duration	Mouse	Muscle	8 × 1600–2000 V/cm, 0.099 ms (19)
	Mouse	Tumor	6 × 1500 V/cm, 0.099 ms (29)
	Pig, monkey	Skin	6 × 1750 V/cm, 0.1 ms (73)
Low voltage	Mouse	Muscle	6 × 100 V/cm, 20–50 ms (51,102)
Long duration	Dog	Muscle	2 × 375 V/cm, 25 ms (16)
	Pig	Muscle	6 × 200 V/cm, 60 ms (17)
	Monkey, rabbit	Muscle	8 × 200 V/cm, 20 ms (103)
	Rat	Skin	8 × 12–24 V/cm, 50 ms (104)
	Mouse	Liver	10 × 150 V/cm, 50 ms (105)
Combination regimen	Mouse	Muscle	1 × 800 V/cm, 0.01–0.1 ms + 4 × 80 V/cm, 83–100 ms (106,107)

degrade the plasmid (21). As animal models, pigs and dogs have muscle fibers that are quite large (37,38) and consequently are more suitable than rodent muscle for electrokinetic enhancement. Polymers such as polyvinylpyrrolidone (PVP) and poly-(L-glutamate) (PLG) at high or low concentrations or mild surfactants in low concentration such as poloxamer 188 have been used to enhance plasmid uptake, with some reduction in tissue damage. Poloxamer 188 may induce sealing of permeabilized lipid bilayers to rescue cells that were not extensively heat damaged; consequently, the expression levels may rise (21,39). Following electroporation of the skeletal muscle of mice, rats, dogs, or pigs (16,40–42), plasmid formulated with PLG or PVP has been observed to increase gene expression up to 10-fold compared with nonformulated plasmid. In mice, preinjection of the electroporated muscle with hyaluronidase, an enzyme that hydrolyzes hyaluronic acid, a ubiquitous component of the extracellular matrix, increases gene expression up to 5-fold with minimal tissue damage (43).

C. Delivery of Plasmid Fragments

Linear fragments of DNA derived from adeno-associated viral vectors have been delivered to the liver by an intra-arterial high-pressure hydrodynamic method, and have been shown to be efficacious and provide long-term expression of a secreted protein (44). Mice injected with a linear DNA expression cassette encoding human α-1-antitrypsin (hAAT) expressed approximately 10- to 100-fold more serum hAAT than mice injected with closed circular DNA over the length of the study.

We examined the effect of electroporation on expression of plasmid fragments containing expression cassettes, with or without residual plasmid backbone in skeletal muscle. There may be many advantages of delivering DNA fragments in vivo from which the antibiotic resistance gene and/or the bacterial origin of replication have been removed. First, the antibiotic resistance gene could render the host organism resistant to that particular antibiotic. In addition, some antibiotic resistance genes, such as the ampicillin gene, contain multiple CpG motifs, which are known to enhance the immune response in muscle cells (45). A less immunogenic vector can reduce the possibility of immune or toxic responses and increase the therapeutic value of the vector (46). In addition, although undocumented for naked plasmid, the possibility of plasmid replication or recombination in vivo exists. It is possible to redesign plasmids with conditional origins of replication, as in the pCOR plasmids (47) or supercoiled minicircles (48) that lack the bacterial origin of replication. The creation of putatively safer plasmid constructs is difficult and will require sufficient resources for the design and testing of new plasmid systems that exceed the overall yield and performance of existing systems.

The pSEAP2 mammalian reporter vector (Clontech, CA) that contains the human placental secreted alkaline phosphatase (SEAP) gene was used in these studies (Fig. 2A). The strong muscle-specific synthetic promoter SPc5-12 (49) was inserted into the pSEAP2 basic vector to create a pSP-SEAP vector. The SEAP coding sequence is followed by the SV40 late polyadenylation signal. The vector backbone also provides an f1 origin for single-stranded DNA production, a pUC19 (prokaryotic) bacterial origin of replication, and an ampicillin (prokaryotic)-resistant gene for propagation and selection in *Escherichia coli*. Several linear plasmid DNA fragments were generated by specific restriction enzyme digestions of the circular DNA. Electrophoresis was used to separate the linear fragments, which were then extracted from the gel. The SEAP gene is an immunogenic protein in most normal adult mammals (50). To avoid an immune reaction against the transgene, studies of the long-term expression of the different non-circular DNA fragments were conducted in severe combined immunodeficient (SCID) mice. On day 1, the mice ($n = 10$ per group) were weighed. Then, their left tibialis anterior muscles were injected with equivalent molarities of DNA fragments or supercoiled plasmid. Of the 6 groups, 1 received uncut, circular DNA; 4 received specific

plasmid fragments; and 1 control group received an injection of phosphate buffered saline (PBS). The injection was followed within 2 min by electroporation, using external caliper electrodes and conditions of 6 pulses, 100 V/cm, 60 ms. A BTX T820 generator (Genetronics, San Diego, CA) was used to deliver square-wave pulses in all experiments. SEAP values were assayed to 54 days postinjection (Fig. 2B). The SEAP expression levels were not different among the treated groups. These results suggest that, when using the in vivo electroporation technique, gene expression, and probably intracellular bioavailability of the plasmid, is not dependent on the plasmid configuration.

III. TISSUE SPECIFIC EXPRESSION

A. Skeletal Muscle

Since the initial report by Aihara and Miyazaki (51), in vivo electroporation of the skeletal muscle for delivery of therapeutic proteins has become widely used. Table 2 summarizes some of the recent literature reports in which electroporation was used to enhance plasmid delivery to skeletal muscle. Although the devices, conditions, methods, and animal models substantially differ, all studies conclude that plasmid injection followed by electroporation can be successfully used to deliver therapeutic genes. The electrokinetic enhancement of plasmid delivery allows the muscle to be used as a bioreactor for the persistent production and secretion of proteins into the bloodstream. The expression levels are increased by as much as 2 to 3 orders of magnitude over plasmid injection alone, to levels comparable to those of adenoviral-mediated gene delivery and may reach physiological ranges. The applications of intramuscular electroporation gene transfer are innovative and intriguing, and span a large array of pathologies: malignancies, renal disease, anemia, prevention of drug toxicity, etc. Thorough review articles address aspects of these proposed therapies (6,52–54). The duration of gene expression was reported to be at least 9 to 14.5 months after in vivo gene electrotransfer into skeletal muscle (55–57). Collectively, these studies provide the evidence that adequate levels of secreted proteins can be achieved using plasmids in a simple, safe, and efficient manner, with significant potential for gene transfer and vaccination for large animals and humans. Interestingly, more and more scientists are now addressing a new problem, the regulation of gene expression, raised by the *adequate* levels of protein production, to maintain levels of expression in concordance with therapeutic needs (15,18,58,59).

B. Liver

The liver represents one of the primary targets for gene therapeutic treatment of numerous metabolic diseases, cancers, hepatitis, and other pathologies. Although recombinant viral vectors have been widely used to introduce new genes into the liver, their usefulness may be mitigated by side effects and potential safety concerns (60–63). Plasmid delivered to hepatic vasculature (64), by hydrodynamic methods (65,66), or by electroporation constitutes an alternative method to deliver transgenes to the liver. Mechanistic studies to characterize electric field distribution for drug or plasmid administration under different conditions were performed in the liver (23). Electroporation is traditionally performed in conjunction with chemotherapy for different malignancies. This treatment, known as electrochemotherapy, has been successful for liver malignancies in animal models (67). Tumor reduction has been recently achieved by locally injecting DNA to the site of interest in the liver followed by the electric field application (see also Table 4). Long-term expression of bcl-xs and tumor regression has been observed after plasmid delivery and electrotherapy using tweezers electrodes (68). In addition, nutritionally regulated foreign gene expression in vivo is attainable locally in the liver by this method (69). A method for efficient gene transfer to the liver by electroporation following tail vein administration of the naked DNA has been recently described (31). According to the authors, systemic injection has the advantage of delivering genes to more hepatocytes when compared with the local injection of plasmid to the liver. These advances in liver gene delivery may provide powerful tools for basic research or potential clinical application studies.

C. Skin

DNA delivery to skin may be useful for the treatment of skin disorders, DNA vaccinations, and other gene transfer applications requiring local or systemic distribution of a transgene product (33). This choice is facilitated by information that shows that electroporation of skin induces a mild and reversible impairment of the barrier function of the skin, a decrease in skin resistance, and a transient decrease in blood flow. Neither inflammation nor necroses is generally observed (70,71). Reporter genes are expressed in the immediate area surrounding the injection site. After direct plasmid injection into skin, transfected cells are typically restricted to the epidermis. However, in different animal species, when electroporation is applied after the injection, larger numbers of adipocytes and fibroblasts and numerous dendriticlike cells within the dermal and subdermal tissues, as well as lymph nodes draining electropermeabilized sites, are transfected (72–74). Compounds such as aurintricarboxylic acid have been found to enhance plasmid expression in rodent, primate, and pig muscles (73). Therapeutic molecules such as erythropoietin or HBV antigen can be efficiently produced by skin cells and remain functionally active up to 7 weeks (34,72).

D. Tumors

Electrochemotherapy, or enhanced delivery of chemotherapeutic drugs, especially bleomycin, to solid tumors has been used successfully for many years (76,77). Clinical trials using this method for the treatment of solid tumors have been conducted in humans and other species (9,78–80). Recently, investigators focused on plasmid delivery to tumors as a means to increase long-term antitumor immunity successfully (7,81,82), to inhibit angiogenesis (83,84), or to reduce tumor volume (85). Interestingly, in some cases, CpG islands within the plasmid backbone may contribute to the antitumor effect in the absence of therapeutic cDNA expression or eukaryotic sequences (86).

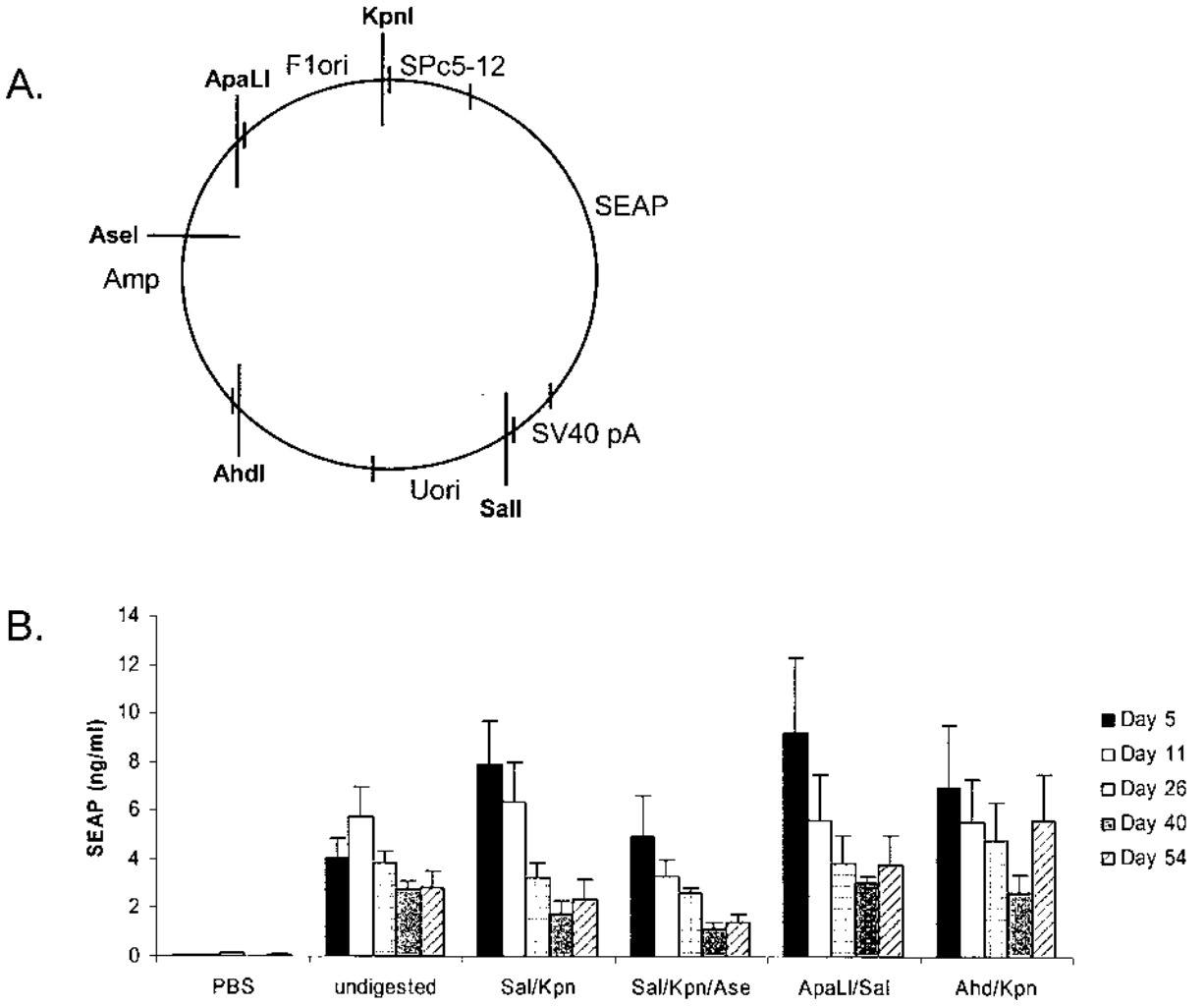

Figure 2 In vivo expression of plasmid fragments delivered by electroporation to the skeletal muscle. (A) The construct pSP-SEAP contains a SPc5-12 synthetic promoter, a human SEAP gene, the SV40 polyadenylation signal (expression cassette), and a plasmid backbone with bacterial replication origin, Uori, an antibiotic resistance gene (ampicillin), and a packaging origin for the SEAP gene, F1ori. Different regions of the plasmid were cut using restriction enzymes to yield the following:

- KpnI/SalI, containing the expression cassette
- KpnI/AhdI, containing the expression cassette and Uori
- ApaLI/SalI, containing the expression cassette and F1 ori
- KpnI/SalI/AseI, containing the expression cassette and other 2 additional fragments of the plasmid backbone.

(B) SCID mice were injected with supercoiled DNA or plasmid fragments. Expression levels were not different among groups.

In a comparative study, 50 micrograms of plasmid was found to be as efficacious as 5×10^9 i.u. adenovirus. Furthermore, adenovirus leakage induced mild to moderate liver damage, while practically no leakage occurred after electroporation (87). Table 3 contains reports that show that improved vectors, delivered using highly effective electroporation methods, may form the basis for future human applications. Updated overviews of the therapeutic perspectives of antitumor drug and DNA electrotransfer are also of significant interest (88,89).

E. Eye

Recently, electric pulse-mediated plasmid transfer has been used to deliver transgenes to cornea in an effort to ensure long-

Table 2 Electroporation for Plasmid Delivery in Muscle

Transgene	Species	Endpoint	Ref.
Restriction endonuclease SmaI	Hamster	Cytochrome C oxidase activity	(108)
Erythropoietin	Mouse	SEAP, erythropoietin levels	(41)
Melanocyte antigen tyrosinase-related protein-2 H-2K(b)-restricted epitope SVYDFFVWL in HLA-Cw3	Mouse	T cells, spleen CD8+T cells, outgrowth of s.c. B16F10M tumors, tumor nodules in i.v. lung metastasis	(109)
GM-CSF, FLT3-L	Mouse	Myeloid, lymphoid dendritic cell expansion, antigen presentation, stimulation mixed lymphocyte	(110)
Neurotrophin-3	Mouse	Cisplatin-induced neuropathy	(111)
Bovine herpes virus glycoprotein D (gD), hepatitis B surface antigen (HBsAg)	Pig	Neutralizing antibody titer, IFN-γ secreting lymphocytes, stimulation index	(112)
Bone morphogenetic protein-4	Mouse	Ectopic calcification or ossification	(113)
Cardiotrophin-1	Mouse	Global weight, lifespan, electromyographic parameters, degeneration of myelinated axons of phrenic nerves	(11)
Fc-fusion-vIL-10	Mouse	IL-10 levels, survival, histology	(114)
Hepatocyte growth factor, GFP	Mouse	Hepatic apoptosis, plasma alanine aminotransferase	(115)
Glial cell line-derived neurotrophic factor (GDNF)	Mouse	Number of motor neurons, survival	(116)
B7-1wa-Ig	Mouse	Autoimmune insulitis, diabetes	(54)
Luciferase, ^{51}Cr-EDTA	Mouse	Relative light units, permeability	(117)
Luciferase	Mouse	Relative light units	(107)
Gastrin, EGFP-N1	Rat, Mouse	Fluorescence microscopy, gastrin level, immunochemistry	(14)
Luciferase	Mouse	Relative light units	(118)
IL-18	Mouse	Image analysis, histochemical changes in plaque composition	(119)
Erythropoietin, GeneSwitch	Rat	Epo levels, hematocrit, immunochemistry	(15)
Luciferase	Mouse	Quantitative imaging	(120)
IL-10	Rat	Survival, myocardial histopathology, hemodynamic parameters	(121)
Laminin alpha2 chain, chimeric dystrophin-EGFP protein	Mouse	Immunohistochemistry	(12)
IL-12, IL-18, luciferase, EGFP	Mouse	Cytokine levels, survival, tumor volume	(122)
IL-1 receptor antagonist (IL-1ra), viral IL-10 (vIL-10)	Mouse	Survival, serum and heart cytokine levels, histopathology	(123)
Human insulin precursor gene	Mouse	Survival, blood glucose, insulinlike protein level	(124)
Luciferase, SEAP, IL-2	Mouse	Relative light units, protein levels	(125)
Erythropoletin	Rat	Hematocrit, protein levels	(104)
Luciferase	Mouse	Relative light units	(13)
Matrix metalloproteinase 4	Mouse	Tumor volume, apoptosis	(126)
Neurotrophin 3	Mouse	Latency of sensory nerve action, immunohistochemistry, serum protein levels	(127)
B7-1, peptides	Mouse	Cytotoxicity, immunohistochemistry	(128)
Luciferase, IL-12	Mouse	Relative light units, cytokine levels	(82)
Luciferase, erythropoietin	Mouse	Relative light units, EPO levels, hematocrit, fluorescence microscopy	(56)
Hepatitis C E2 ectodomain with N-terminal fusion with hypervariable region 1 (HVR1) peptide mimic	Mouse	Titers, cytotoxicity, seroconversion	(129)
EGFP-dystrophin fusion proteins	Mouse	Fluorescence microscopy	(130)

Table 2 Continued

Transgene	Species	Endpoint	Ref.
HIV gag, luciferase	Mouse	Luciferase mass, titer	(131)
Luciferase, β-galactosidase	Rat	Histochemistry, relative light units	(132)
β-galactosidase, luciferase	Mouse	Histochemistry, titer, luciferase mass, confocal microscopy	(133)
Hepatitis B surface Ag	Mouse, rabbit, guinea pig	Antigen level, titer, cytotoxicity	(134)
Luciferase	Mouse	Relative light units, ^{51}Cr-EDTA uptake	(106)
Erythropoietin	Rat	RT-PCR, Epo levels, hematocrit	(135)
β-galactosidase, GFP	Rat	Histochemistry, microscopy	(22;55)
Tissue inhibitor of metalloproteinases-4	Mouse	Immunohistochemistry, tumor size, Western blot	(136)
SEAP	Mouse	SEAP levels, antibody titer	(50)
IL-12	Mouse	IL-12 levels, tumor volume, flow cytometry	(137)
Influenza B hemagglutinin, neuraminidase, nucleoprotein	Mouse	Survival, antibody titer, body weight	(138)
MyHC promoter family, luciferase	Mouse	Fold change in relative light units	(139)
Erythropoietin	Mouse	EPO levels, hematocrit	(140)
INF-α, luciferase, SEAP, IL-2, IL-12, endostatin	Mouse	Tumor volume, tumor vascularization, luciferase mass, SEAP mass	(141)
Luciferase (as plasmid and in adenovirus vector)	Mouse, rat	Relative light units, histology	(87)
INF-γR/Fc fusion protein	Mouse	INF-γ levels, survival, IgG subclass levels, BrdU incorporation in T cell subset, immunohistochemistry	(142)
Luciferase, β-galactosidase,	Mouse	Relative light units, histochemistry	(143)
Luciferase, β-galactosidase, FGF1	Mouse, rat, rabbit, Maccaca	Luciferase mass, histochemistry, immunohistochemistry	(103)
Erythropoietin, SEAP, β-galactosidase	Rat, mouse, rabbit	Histochemistry, SEAP, EPO levels, hematocrit, kidney function	(144)
Erythropoietin	Mouse	EPO levels, hematocrit	(59)
gp100, TRP-2	Mouse	Tumor rejection, cytolysis, survival	(145)
Erythropoietin	Rat	Epo levels, hematocrit, platelet count, blood pressure, weight	(104)
LFT3 llgand, GM-CSF	Mouse	LFT-3, GM-CSF levels, splenocytes, splenic DC	(110)
Erythropoietin	Mouse	qPCR, Epo levels, hematocrit, erythrocyte membrane parameters	(146)
Hepatocyte growth factor	Rat	Renal function, histology, immunohistochemistry, plasma HGF levels, HGF mRNA levels	(147)
M. tuberculosis antigens mpb79, ag85b, ag85a, β-galactosidase	Mouse	Antigen-specific $CD4^+$, $CD8^+$ T cells, IFN-γ, IgG subclasses	(148)
Luciferase, β-galactosidase	Mouse	Luciferase mass, histochemistry	(149)
Laminin α2, chimeric dystrophin-EGFP, β-galactosidase	Mouse	Immunohistochemistry, fluorescence microscopy, histochemistry	(130)

lasting expression of a desired protein either for treatment of a local disease or for research purposes. Results of these studies demonstrate that electroporation is an excellent method for delivering genes to multiple cell layers within the cornea with extremely high levels of gene expression and little, if any, inflammatory response or tissue damage (90,91). Retinal ganglion cells have also been targeted. After a first demonstration showing retrograde labeling of up to 41% of the total ganglion cells in the electroinjected area (91), other studies with brain-derived neurotrophic factor (BDNF) gene transferred by in vivo electroporation showed protection of axotomized retinal ganglion cells against apoptosis (91,92).

Table 3 Electroporation for Plasmid Delivery in Tumors

Transgene	Species	Endpoint	Ref. Citation
CpG (luciferase)	Mouse	Tumor volume	(86)
IL-12	Mouse	Tumor volume	(7)
Antisense VEGF, sFlk-1	Mouse	Tumor volume	(83)
GFP	Mouse	Tumor area	(150)
HSV*tk*	Mouse	Tumor volume, histopathology, metastasis incidence	(85)
Tie2 receptor	Mouse	Tumorigenicity with neovascularization	(151)
TRAIL/Apo2L	Mouse	Tumor volume, histopathology, western blotting of serum	(152)
IL-2, GM-CSF	Mouse	Tumor volume	(8)
Luciferase	Mouse	Relative light units	(153)
IL-12	Mouse	Tumor volume	(154)
IL-2, IL-12	Mouse	Tumor volume, protein levels	(155)
IL-12, luciferase	Mouse	Relative light units, serum cytokine levels, tumor volume, histochemistry	(105)
GM-CSF, IL-2	Mouse	Tumor volume	(81)
Luciferase, β-galactosidase	Mouse	Luciferase mass	(156)
Luciferase,	Mouse	Relative light units	(157)
Luciferase, EGFP, diphtheria toxin fragment A	Mouse	Relative light units, histopathology	(158)
HSV-*tk*, GFP, diphtheria toxin fragment A	Mouse	Tumor volume, microscopy, histopathology	(159)
Endostatin, luciferase	Mouse	Relative light units, tumor volume, endostatin levels, number of metastases	(84)
Dominant-negative Stat3	Mouse	Histochemistry, tumor volume, histology	(160)
β-galactosidase	Mouse	Histochemistry	(161)

F. Others—Cardiac Muscle, Vascular Smooth Muscle, and Testes

Investigators have demonstrated the potential application of plasmid electroporation in many other tissues (Table 4). For example, in the cardiac tissue of chicken (93) green fluorescent protein (GFP) levels achieved by electroporation were similar to 10^6 transduction units/mL of adenovirus GFP. Smooth muscle cells in the renal tubulointerstitial compartment were also targeted. Myocyte-enriched calcineurin-interacting protein, (MCIP1), inhibits cardiac hypertrophy in vivo (75). In vivo injection of a plasmid expressing an enzyme for early growth response gene 1 via the urethra followed by tweezer electroporation prevented interstitial fibrosis in a rat model of kidney obstruction (94). Although gene transfer via in vitro fertilization with directly injected oocytes is a widely used method, recent studies suggest that in vivo electroporation technique in conjunction with direct plasmid injection into testes could be used to create transgenic animals or to treat some types of infertility. After testicular injection and electroporation, plasmid is rapidly transferred to epididymal ducts, and incorporated by ductal epithelial cells and epididymal spermatozoa (95). Long-lasting GFP expression can be detected in the spermatogenic cells even 2 months after electroporation. Natural mating with normal adult females revealed that 65% of the transfected males remained fertile and could generate their offspring normally (96). Infertile mice expressed an enhanced green fluorescent protein (EGFP) construct in the Sertoli cells in the testis for at least 35 days (97).

G. Embryo

A major challenge in current developmental biology and neuroscience is to understand the functions and activation sequence of thousands of genes involved in organogenesis, plasticity, physiology, and function. An unexpected tool was provided by the electroporation technique. Using this method, it is now possible to express foreign genes or antisense sequences in either a single cell or population of cells in vivo, by varying the size and type of electrodes or by modifying the pattern of stimulation. Recent advances are summarized in Table 5 and point out that electroporation is one of the most versatile of the nonviral technologies.

IV. FUTURE DIRECTIONS

All the studies presented above describe electroporators and methods based on a predetermined voltage between the electrodes. Because the impedance between electrodes that are

Table 4 Electroporation for Plasmid Delivery in Various Organs

Transgene	Organ	Species	Endpoint	Ref.
Luciferase	Skin	Mouse	Relative light units	(33)
Erythropoietin	Skin	Rat	Epo levels, hematocrit	(34)
Biophysical methods, histology	Skin	Rat	Transepidermal water loss, chromametry, impedance and laser Doppler velocimetry or imaging measurement	(70)
EGFP	Skin	Mouse	Confocal microscopy	(71)
Luciferase, IL-2	Skin	Mouse	Relative light units, serum protein levels	(162)
HBV sAg, luciferase, EGFP, β-galactosidase	Skin	Mouse	Relative light units, histochemistry, titer	(72)
Luciferase, EGFP, β-galactosidase	Skin	Rat, macaque, pig	Luciferase mass, histochemistry	(73)
EGFP	Skin	Sheep	Microscopy	(74)
Luciferase	Skin	Mouse	Relative light units, histochemistry	(163)
Pro-opiomelanocortin	Spinal cord	Mouse	Elevated neuropathic pain threshold	(164)
GFP	Spinal cord		Fluorescent microscopy, RT-PCR, Western blotting	(165)
GFP, DsRED, morpholino oligonucleotides	Brain, retinal ganglion cells, single neurons	*Xenopus levis* tadpole	Imaging, dendritic arbor growth	(166)
Protein-disulfide isomerase	Brain	Rat	RT-PCR, immunohistochemistry, apoptosis	(167)
Engrailed, VP16-engrailed, mutant engrailed [En-2(F51rE)]	Brain	Chicken	Histochemistry	(168)
Embryonic lethal Hu proteins, GFP	Brain	Chick	Immunohistochemistry, microscopy	(169)
caNotch, Hes1, DsRED, EYFP, ECFP, d2EFP,	Embryonic brain	Mouse	Imaging, in situ hybridization	(170)
Cadherins, GFP	Tectal projection neurons	Chick embryo	Differential axonal pathfinding	(171)
EGFP, DsRED	Neuron	Tadpole	Fluorescence microscopy	(166)
Influenza hemagglutinin	Nasal mucosa, muscle	Mouse	Survival, CTL assay, antibody titer, viral infective dose	(172)
EGFP	Retinal ganglion cells	Rat	Fluorescence microscopy	(91)
Brain-derived neurotrophic factor	Intravitreal delivery	Rat	Survival of axotomized retinal ganglion cells	(92)
GFP	Corneal stroma	Rat	Relative fluorescence, RT-PCR	(173)
EGFP, luciferase	Synovium	Rat	Relative light units, cell numbers	(174)
β-galactosidase	Eye	Rat	Histochemistry	(173)
Tbx5, GFP	Eye	Chick	Histochemistry, fluorescence microscopy	(175)
β-galactosidase	Eye	Rat	Histochemistry, histology	(176)
Luciferase	Testis	Quail	Relative light units	(177)
Luciferase, β-galactosidase	Kidney, testes, bladder	Rat	Relative light units, histochemistry, RT-PCR	(178)
β-galactosidase, CAT	Bladder	Mouse	Histochemistry, enzyme activity	(179)
β-galactosidase, neuronal e-NOS	Penis	Rats	Histochemistry, erectile response	(180)
Alkaline phosphatase	Oviduct	Chicken	SEAP levels in egg white	(181)
CAT, luciferase	Oviduct	Chicken	Relative light units, activity	(182)
Luciferase, β-galactosidase	Liver	Mouse	Relative light units, histochemistry	(31)
bcl-xs, CAT	Liver	Rat	Histochemistry, tumor size, nodule number, apoptotic index	(68)
Luciferase	Liver	Mouse	Imaging, relative light units	(69)
GFP	Liver	Rat	Imaging microscopy	(183)
Human growth hormone	Udder	Sheep	hGH levels in milk	(184)
Luciferase, EGFP	Mesenteric arteries	Rat	Luciferase mass, histochemistry, fluorescence microscopy	(185)

Table 5 Electroporation of Embryos

Transgene	Species	Endpoint	Ref.
Truncated Pax6 fused to Drosophila engrailed repressor domain, GFP	Rat	Imaging, antibody staining of Pax6, Islet1/2, Nkx2.2, Wnt7b, Hu protein	(186)
Winged-helix transcription factor FoxD3, fusion protein with EGFP	Chick	In situ hybridization, fluorescence microscopy, immunohistochemistry	(187)
Sonic Hedgehog	Chick	Whole mount in situ hybridization, histochemistry	(188)
GFP	Chick	Microscopy	(189)
Interfering RNA, GFP, β-galactosidase	Mouse	Fluorescence microscopy, histochemistry	(190)
Slug, GFP	Chick	In situ hybridization, Immunohistochemistry, fluorescence microscopy	(191)
EGFP, N-cadherin-EGFP fusion construct, DsRED	Chick	Confocal and 2-photon microscopy	(192)
Pax-5, EGFP, β-galactosidase	Chick	Histochemistry, in situ hybridization	(193)
Pdx-1, ngn3, β-galactosidase		Histochemistry, in situ hybridization, immunohistochemistry	(194)
Krox-20, β-galactosidase	Chick	Histochemistry, in situ hybridization, immunohistochemistry	(195)
β-galactosidase	Chick	PCR	(196)
dsRNA against Otx2, Foxa2, β-galactosidase	Mouse	Histochemistry, in situ hybridization, immunocytochemistry, histology	(197)
R-cadherin, cadherin-6, GFP	Mouse	Fluorescence microscopy, Immunohistochemistry	(198)
β-galactosidase, EGFP	Chick	Histochemistry, fluorescence microscopy	(199)
EphA4/ephrin-A5, EGFP	Mouse	Immunocytochemistry, confocal imaging	(200)
EGFP	Mouse	Fluorescence microscopy, histology	(201)
Pax6, EGFP	Rat	Fluorescence microscopy, immunocytochemistry	(202)
Tlx, fusion genes Tlx-DNA-binding domain–engrailed repressor domain, TlxVP16 activation domain	Chick	Histochemistry, in situ hybridization	(203)
Six3 and deletion mutants, Grg5, luciferase, CAT	Chick	In situ hybridization, immunohistochemistry, CAT activity	(204)

embedded in a tissue can vary from case to case, or tissue to tissue, a predetermined voltage may not necessarily produce a predetermined current (98). As an example, Fig. 3 depicts a direct comparison of the 2 methods in the pig muscle. Notice that a predetermined voltage pulse causes an increase in the current flowing through a porcine muscle tissue during the duration of the pulse with the loss of a perfect square wave; in contrast, a constant current source actually maintains a constant current through a porcine muscle tissue. Thus, in many cases, the experiments do not provide a means to delineate the exact amount of current to which the cells are exposed. For this reason, conventional electroporators may generate amounts of heat in tissues that can easily kill cells (99,100). For example, a typical electronic 50-mS pulse with an average current of 5 Amperes across a typical load impedance of 25 ohms can theoretically raise the temperature in tissue 7.5°C, which is enough to kill cells. The physics of tissue injury caused by electrical shock is reviewed by Lee et al. (101). In contrast, the power dissipation decreases in a constant current system and prevents heating of a tissue, which may reduce tissue damage and contribute to the overall success of the procedure. Thus, there is a need to overcome the technological problems associated with constant voltage electroporation by providing a means to control effectively the amount of electricity delivered to the cells. This can be accomplished by precisely controlling the ionic flux that impinges on the cell membrane conduits. A constant current electrokinetic device has been tested in our laboratory, in collaboration with Robert H. Carpenter, DVM, MS. The paired-needle electrodes that use a predetermined voltage pulse across opposing electrode pairs creates a centralized pattern during an electroporation event in an area where congruent and intersecting overlap points develop. This area can be visualized as an asterisk pattern, as shown in Fig. 3C. However, asymmetrically arranged needle electrodes without opposing pairs will produce a decentralized pattern during an electroporation event in an area in which there are no congruent electroporation overlap points. One example of such symmetry is shown in Fig. 3D, with a decentralized pattern area of electroporation that resembles a pentagon. In this case, plasmids would be stereotactically

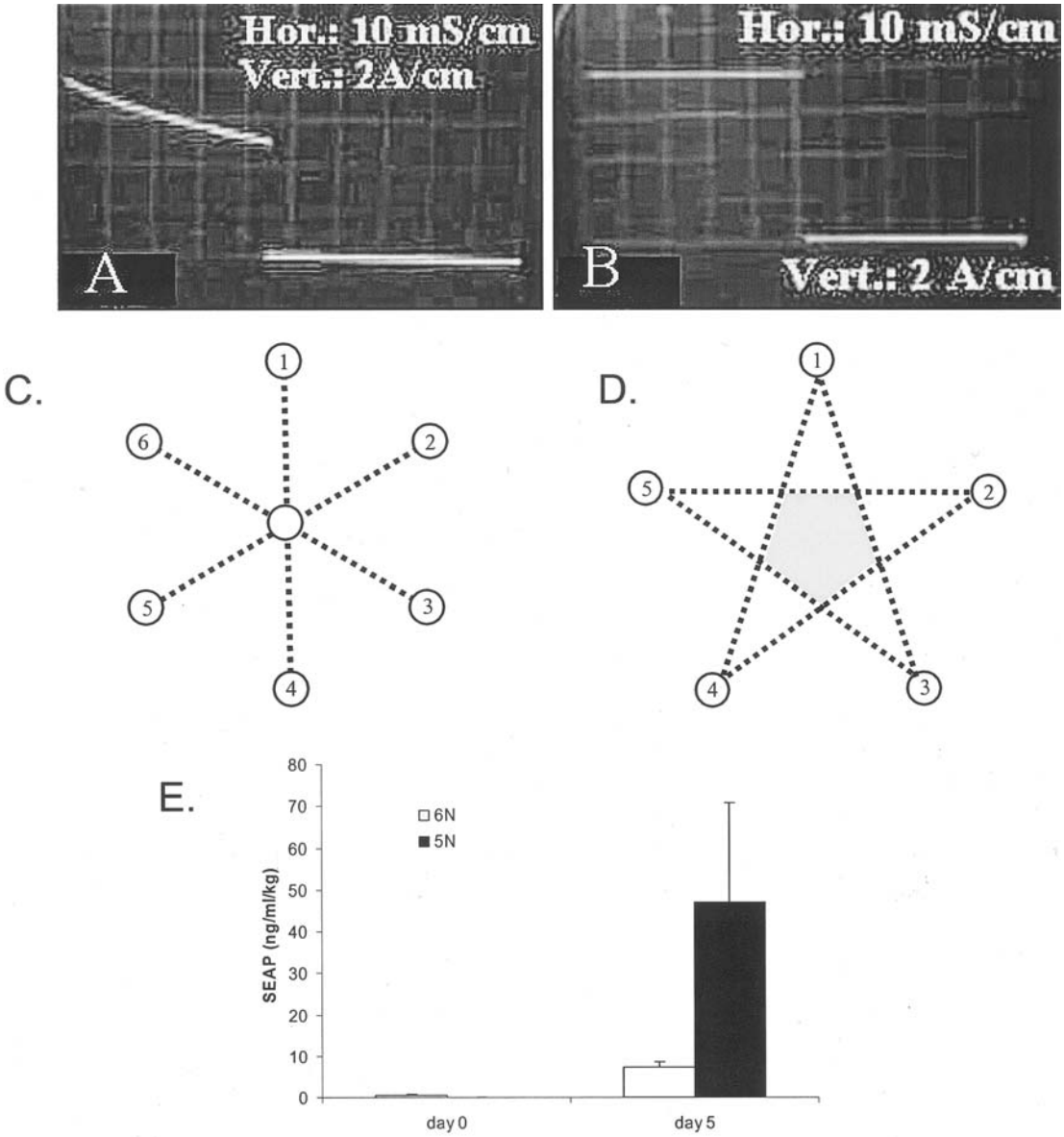

Figure 3 Constant voltage electroporation with paired needles vs. constant current electroporation using a pentagonal electrode array. (A) A predetermined voltage pulse causes an increase in the current flowing through a porcine muscle tissue during the duration of the pulse. In contrast, (B) a constant current source actually maintains a constant current through a porcine muscle tissue. (C) Maximum intensity electric field after constant voltage electroporation using a paired needle array. (D) Diffuse electric field after constant current electroporation using a pentagonal needle array. (E) Comparison of gene expression between the 2 delivery methods using similar electric field intensities.

delivered in-between the internal electrodes, followed by the constant current electric field pulses. A comparison of in vivo results obtained with a classical electroporator and a constant current device with asymmetrically arranged electrodes in adult pigs is depicted in Fig. 3E.

V. SUMMARY

The electrokinetic enhancement of plasmid delivery has become widely used for a variety of academic and preclinical applications. Since 1998, more than 90 transgenes have been

used to achieve quantitatively significant in vivo endpoints in response to the circulating levels of their respective protein products. The full potential of skeletal muscle as a bioreactor for production of therapeutic proteins has yet to be realized. It is well established in animal models that tumor growth can be inhibited by transgenes expressed either locally by expression in tumors or systemically in remote muscles. This technology holds promise for significant advances in treatment of tumors and their associated metastatic disease. Developmental biologists have recognized the experimental power of electroporation to augment plasmid delivery to single cells or populations of cells at stages of development, as selected by the investigator. Electrokinetic enhancement of plasmid delivery, particularly in skeletal muscle and embryos, is a significant advance toward the development of practical uses envisioned for the molecular information contained in the mammalian genomes.

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15

Gene Gun Technologies: Applications for Gene Therapy and Genetic Immunization

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I. INTRODUCTION

Biolistic gene delivery (a.k.a. microparticle injection, ballistic, bioballistic, particle bombardment, etc.) is mediated by devices generally known as ''gene guns'' because these devices literally propel nucleic acids on ''bullets'' to ''shoot'' genes into cells. Chemicals, proteins, mRNA, or DNA are first coated on dense metallic particles of 0.25 to 5 μm in diameter, and these are accelerated into target cells or tissues to deliver these substances directly inside cells. Unlike the cooperative processes needed for gene delivery by viral or nonviral vectors (Fig. 1A), biolistic gene delivery (1) breaks down many of the barriers to transfection by applying kinetic energy to drive macromolecules across cell membranes into the cell interior (Fig. 1B). Direct delivery into the cell cytoplasm avoids the necessity for cell receptor binding, engagement of receptor-mediated endocytosis mechanisms, and escape from endosomes (Fig. 1B). As such, biolistic transfection using gene guns allows for transfection of cells normally refractory to transfection. This method also avoids destruction of nucleic acids by nucleases present in extracellular fluids and in the endosomes (2), thereby allowing gene delivery without the necessity to package nucleic acids into virions or nonviral particles. Given these features, gene guns have become a potent method for gene delivery into cells in vitro and for gene delivery directly into animals and humans, thereby allowing application of simple plasmids for gene therapy and genetic immunization applications.

II. BRIEF HISTORY OF BIOLISTICS AND GENE GUNS

Biolistic technology was originally developed in the area of plant genetics to introduce genes into target cells that were largely refractory to traditional methods of gene delivery (1). Biolistic lore holds that the gene gun was inspired by interactions between edible bonsai plants and squirrels, and the later involvement of an air rifle to control these interactions. Legend has it that in the course of those events, Dr. John Sanford made the pivotal extrapolation that if an air rifle could be used to deliver macrobullets into a macrotarget, perhaps another gun could be used to deliver microbullets into microtargets like living cells. By extension, if a microbullet could be shot into living cells without killing them, could a microbullet also be used to carry nucleic acids into those cells to genetically modify them?

The obvious answer now to this strikingly simple but powerful question is, of course, ''yes.'' However, moving from concept to a practical device that could deliver DNA into cells without killing them required a number of years of work by Sanford's group and several other groups. Sanford's group originally focused on biolistic gene delivery for plant and prokaryotic applications (1,3,4). These efforts developed a wide variety of gene guns, including the precursor to the gunpowder-driven commercially available gene gun from BioRad. Further gene gun development occurred as a collaboration between John Sanford and Stephen Johnston, who did their graduate training together in Bob Hanneman's laboratory at

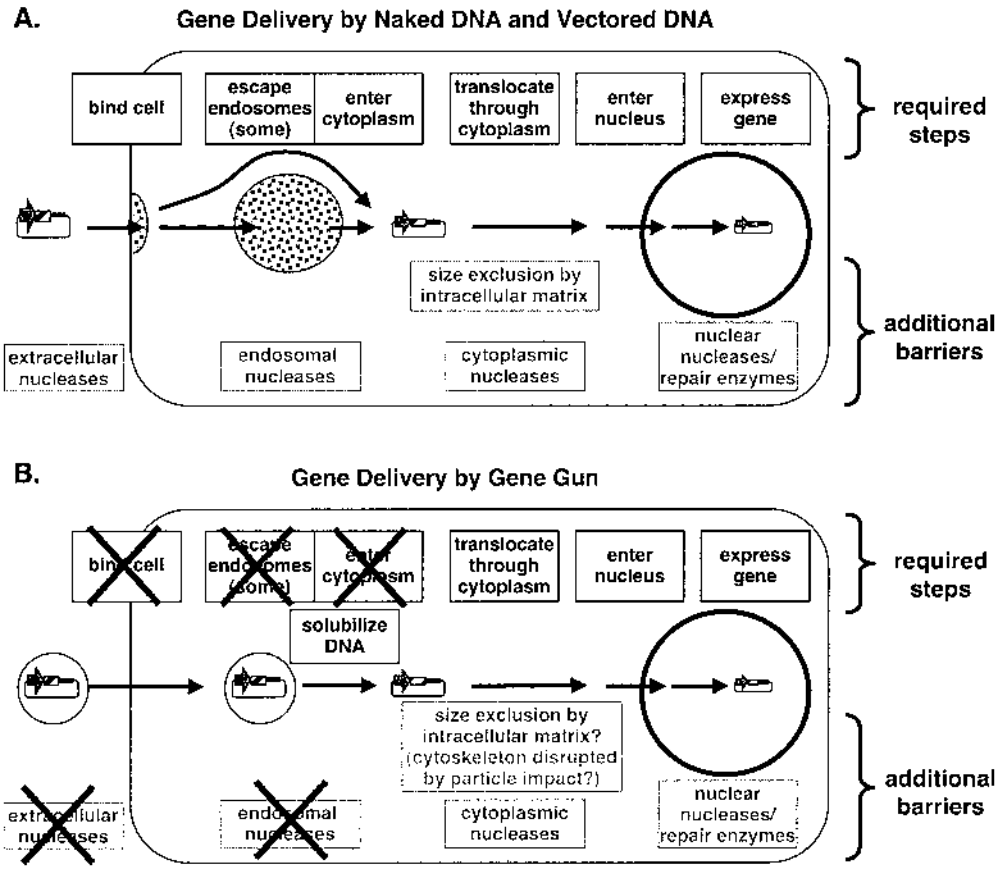

Figure 1 Cartoon of the required events and theoretical barriers to gene delivery in mammalian cells. (A) Gene delivery by naked plasmid DNA and viral or nonviral gene delivery vectors. (B) Gene delivery by gene guns using kinetic energy to overcome some required events and barriers.

the University of Wisconsin. This collaboration between Sanford's group and Johnston's group enabled a number of new gene guns, including a helium-driven vacuum chamber device (Fig. 2A) that was the predecessor of BioRad's helium-driven PDS1000™ for mammalian cell and animal transfection. This collaboration along with Rumsey–Loomis generated several handheld helium-driven guns (Fig. 2B) for animal transfection including the infamous ''bazooka'' and the ''wand'' (Fig. 3). The long-standing Sanford–Johnston collaboration also provided first demonstration of mitochondrial transformation by biolistics, as well as early demonstrations of transfection of mammalian cells and living animals in collaboration with Sandy Williams (5–7).

Following Sanford's first demonstration of gene gun technology (1), several other groups developed parallel or alternate gene gun technologies for biolistic gene delivery [reviewed in (7)]. Most notable of these are the efforts by the group from Agracetus (now Powderject), who developed a series of gene guns, including the electrically driven Accell™ device (8), and an entrainment gene gun (Fig. 2C) that was the ultimate prototype for the commercially available Helios™ device (BioRad).

Commercialization of the Helios gene gun was an important milestone for enabling the use of biolistics for gene therapy because it opened the technology up for those who previously could not engineer their own gun or who could not obtain one of the few spare guns from established groups. This product was also important because each ''home-built'' gene gun was different from every other one, so it was nearly impossible to standardize methods or compare efficiencies between different groups. One final advantage of the Helios gun relative to most home-built guns is that it is a 12-shot ''revolver'' gene gun allowing 12 transfections before the gun needs to be reloaded. In contrast, nearly all other gene guns were single-shot guns requiring reloading after each shot. Because each ''reload'' on older gene guns generally took about 1 minute to perform, the ability to more quickly reload the 12-shooter Helios and perform 12 transfections in a row greatly facilitated transfections of large numbers of cells or animals.

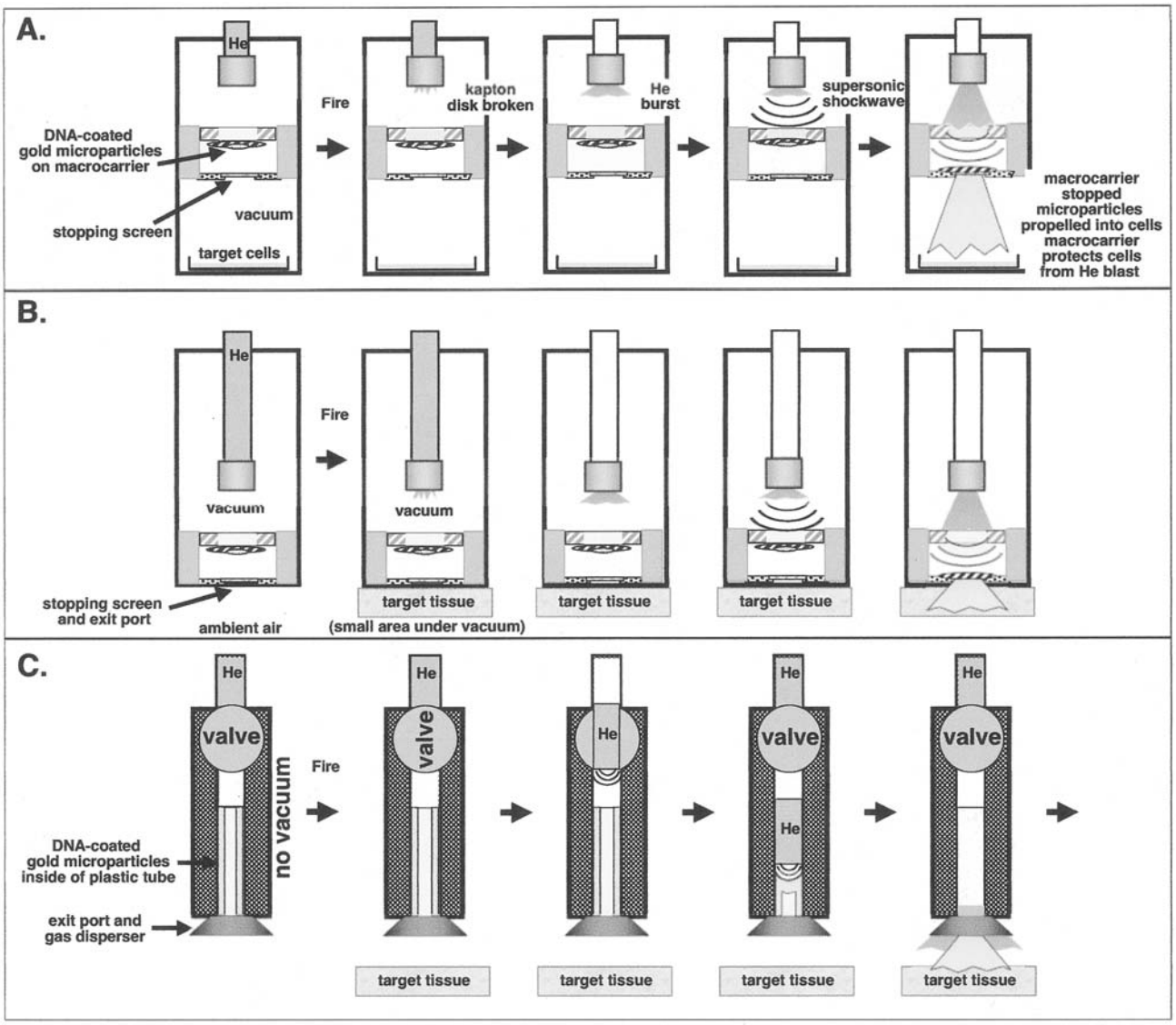

Figure 2 Cartoon of the particle acceleration by different types of gene guns. (A) Helium-driven chambered gene gun under vacuum. (B) Helium-driven handheld gene gun with internal vacuum. (C) Entrainment gene gun. See the color insert for a color version of this figure.

Sanford's pivotal incite that produced the gene gun revolutionized genetic engineering of a number of plant species, including soybeans and corn [reviewed in (9)]. Subsequent work demonstrated that biolistic gene guns could deliver genes not only into plant cells, but also into bacteria, yeast, and fungi [reviewed in (9)]. Biolistics was also the first technology able to genetically modify subcellular organelles, including chloroplasts (10) and mitochondria (11).

This work in other organisms laid the foundation for the application of gene guns for mammalian cells and intact animals. The first published demonstration of mammalian cell modification by biolistics was by Zelenin et al. demonstrating gene delivery into cultured mouse 3T3 cells in 1989 (12). This was followed by in vitro and in vivo gene gun-mediated gene delivery by groups from Agracetus published in 1990 (13) and by the Sanford–Johnston–Williams collaboration in 1991 (14). In the 1980s, these efforts to deliver plasmid DNA into living animals by gene gun occurred parallel to efforts by Jon Wolff's group to deliver plasmids as naked DNA into skeletal muscle by injection, which were published in 1990 (15). Prior to these observations, in vivo gene delivery could routinely be performed only by use of complex nonviral or viral vectors

Figure 3 Prototype handheld gene guns developed in the Johnston Laboratory. (A) The bazooka. (B) The wand.

for gene delivery. The ability to deliver simple plasmids into living cells in living animals by gene gun and by naked DNA injection were pivotal technological advances that enabled a variety of gene therapy applications. They particularly allowed one to transfect living animals or humans with vectors that were unencumbered vector chemicals or antigens.

III. THE MECHANICS AND BIOLOGY OF BIOLISTIC TRANSFECTION

A. Microparticles

The need for ''bullets'' or microparticles in this technology relates to the fact that kinetic energy depends on both mass and velocity ($p = m \times v$). Therefore, applying velocity to a low mass nucleic acid has a low likelihood of imparting sufficient kinetic energy to the molecule to drive it through the membrane and protein structures of a living cell. To circumvent this, nucleic acids and proteins are attached to dense carriers usually in the form of microparticles or ''microbullets'' with diameters from 0.25 to 5 μm (1). Metal microparticles are the carrier of choice for biolistic gene delivery because they have unparalleled density to maximize cell and tissue penetration while minimizing the size of particle needed to achieve this mass. A variety of metals including tungsten and depleted uranium, have been used for biolistics (1,5–7,14). Although any metal could theoretically be used, gold is the favored microparticle material for most mammalian applications because this metal is both dense and inherently biocompatible. For most applications, particles of 1 to 3 μm are used because these are sufficiently small relative to most mammalian cells to allow the particle to fit in the cytoplasm, but they are also sufficiently large to penetrate through 10 to 20 cell layers when applied in vivo (13,14). For the cornified epithelium of the skin, this size of particle can penetrate into the epidermis and dermis, although efficient gene delivery is usually observed only in the epithelial layers (Fig. 4). Although larger particles can be used, these typically do not bind DNA well and frequently cause more tissue damage than transfection and so are generally not used.

The gold microparticles used for biolistics are usually not made for this specific application, but are made as a by-product of other manufacturing processes. As such, obtaining ''good'' particles of the right size has historically been difficult and required screening of each batch of particles to (1) confirm they were the correct size, (2) that they were spherical and monodisperse, (3) that they could efficiently bind DNA, and (4) that they mediated transfection. Generally, if the particles satisfied the first 3 requirements, they would function well for transfection. Transfection efficiency generally correlates with the ability of the particles to bind DNA. Particles that bind 50% less DNA per particle generally mediate 50% less transfection. In practice, DNA binding after precipitation in high calcium varies substantially between different gold particles from different vendors. Given this, investigators would usually screen a small batch of a given lot and then buy as much of that lot as they could afford with typical costs on the order of $300 to $400 per gram. BioRad supplies gold particles that they have screened for activity for the gene gun. These cost considerably more per unit gold, but do save the investigator the necessity to screen and buy large amounts of particles.

B. Precipitation of DNA onto Microparticles

Nucleic acids and proteins do not inherently bind to gold particles. A number of methods have been tested to attach nucleic

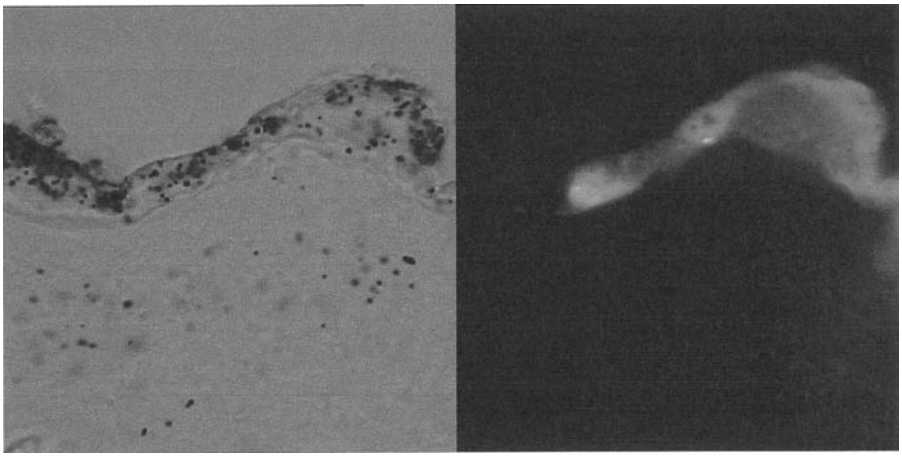

Figure 4 Cross-section of mouse skin transfected with the gene gun. The panel on the left in black and white shows the tissue and locations of the particles as dark dots. The panel on the right shows epidermal expression of green fluorescent protein (GFP) after delivery of its gene on the particles. Note, expression is restricted to the epidermis, despite the fact that particles have penetrated deeper into the dermis. See the color insert for a color version of this figure.

acids and proteins to particles. Although one could covalently attach DNA or RNA to particles or use very strong noncovalent methods, the most common method involves preciptation of DNA onto the particles using high concentrations of calcium in the presence of polyamines or polymers to protect the DNA (1,5,6). Simple precipitation is ideal for this approach due to the need for ''reversibility'' when delivering nucleic acids by any method. Producing a vector or a gene gun particle that is essentially like a rock might provide robust protection from physical or nuclease damage, but a nucleic acid ''rock'' is unlikely to mediate transfection because it would remain trapped after entry into the cell. For most nonviral vectors, this need for reversibility is solved by relying heavily on charge interactions to assemble negatively charged nucleic acids with positively charged chemicals such as a cationic lipids or peptides. For the gene gun, a gold particle has little charge or binding affinity for nucleic acids. Therefore, to satisfy the attachment need and the reversibility problem, the simplest and most robust solution is to precipitate the DNA in the presence of 1.5 M $CaCl_2$ in the presence of spermidine or another polymer. By this approach, the nucleic acid becomes insoluble in the high calcium and precipitates onto the gold particles in suspension. The DNA-calcium precipitate is ''sticky,'' so it adsorbs to the particles efficiently. As the particles are loaded with precipitate, they tend to settle out of solution faster than unloaded particles. The gold particles are big enough that they will settle out of suspension normally, but this is accelerated when they are coated with nucleic acids. A good indication that nucleic acids have successfully attached to the particles is that they will have a tendency to stick together somewhat and will need to be triturated (pipetted repeatedly) to be brought back into a state that is easily resuspended. A good indication that the ratio of nucleic acid to particles is too high is that the particles will form a fairly solid ''clump'' at the bottom of the tube. If observed, too much DNA was added. If not too far gone, the clumps can in some cases be broken apart by trituration, although the resulting particles will likely consist of aggregates of multiple particles that are more likely to cause cell/tissue damage. The particles are then washed in 70% ethanol, then dried in 100% ethanol. The microparticles are then transferred to a macrocarrier, such as a plastic bullet (1) or a plastic disc (7,8), or they can be coated on the inside of a tube for entrained delivery (3) (Fig. 2) in dry 100% ethanol. For macrocarrier-driven guns, transfer of the particles in dry 100% ethanol allows the ethanol to evaporate off the suspension (in a dessicator box) such that the nucleic acid slurry dries and adsorbs to the macrocarrier with sufficient adhesion to remain attached while at rest, but with low enough adhesion that they will release when impacting a stopping plate or screen (Fig. 2A). Working out the minute variations that allowed nucleic acid adhesion to particles and particle adhesion to macrocarriers required substantial effort by early investigators and were pivotal to enabling the gene gun technology.

C. The Effects of Humidity

One critical aspect to the preparation of DNA-coated microparticles is performing DNA precipitation and storage in a low-humidity environment. For the Sanford–Johnston group, this parameter was discovered only after a few years of frustration where they observed that they could get efficient gene delivery for 6 months and then transfection efficiency would drop for the next 6 months. This cycle continued until they realized that the gun worked well in the winter, but poorly during the humid summers in North Carolina at Duke Univer-

sity (Stephen Johnston, personal communication). The poor transfection appeared to be due to the fact that DNA-coated microparticles loaded on macrocarriers in a humid environment did not release or ''unstick'' from the macrocarrier efficiently, whereas particles prepared in a dessicator with dry reagents released well and mediated efficient transfection. This humidity variable was unobserved prior to this because Sanford's work was performed in upstate New York, with a considerably lower humidity than North Carolina in the summer. Once only dry reagents were used (e.g., 100% ethanol stored in a dessicator), and macrocarriers and DNA-coated particles were prepared in a dessicator flooded with dry helium gas, efficient transfection was routine at any time of the year.

Particle preparation guidelines for the Helios device are similar to those described with the exception that polyvinyl pyrolidone (PVP) is added to the precipitation reaction to increase the ability of the particles to stick to the inside of the plastic tube for this entrained mode of particle acceleration (Fig. 2C). For this system, the use of too much PVP gives the same problem as humidity in which the particles will not release efficiently from the tube. Although BioRad holds that humidity does not affect their system, in Houston, Texas (which is probably even more humid than Duke), we observed variations in the release of particles from the Helios that may be due to humidity effects. Given this, we prepare and store particles for the Helios using dessicated 100% ethanol, dry the particles with dry N_2 gas, and store them in a dessicator.

D. Particle Acceleration

Once particles are attached to their macrocarrier or lined on the inside of their tube for entrainment, they are ready to be accelerated. Kinetic energy can be applied to the macrocarrier or the microparticles directly from a variety of sources, including gun powder (1), and high-pressure gases, including nitrogen (12), air (16), and helium (7), and by electrical discharge to vaporize a droplet of water explosively (8). Efficient acceleration of microparticles for most guns does not occur simply by ''blowing'' the particles into the target tissue, but appears to require the production of a supersonic shockwave by the explosive release of gases, whether the energy source is gunpowder, gas, or electrical [(7) and Figs. 2A and B]. Shockwave efficiency appears to increase with decreasing molecular weight of the gas. For this reason, helium is the current propellant of choice for most gene guns. Although hydrogen might be more efficient than helium, the risk of combustion and explosion by this gas precludes its use.

Creating a supersonic shockwave requires the explosive release of gas or vapor. Generating this much force with helium generally employs gas pressures of 1000 to 2000 psi to create sufficient explosive energy to produce shockwaves. Release of this much gas is comparable to the force generated by a high-powered rifle and, if unrestrained, this gas blast will deafen anyone the room. This amount of gas will also destroy any cells in the path of the blast. Given this, much of the early development of gene guns involved a balancing act of generating sufficient force to produce a shockwave, while engineering a robust mechanism to protect the target from the gas blast used to produce the shockwave. One part of the solution to this problem was to generate the gas blast in a vacuum (Figs. 2A and B). Shooting in a vacuum not only improved shockwave production, but also absorbed some of the gas blast after gene gun firing. In addition, removal of ambient air prior to firing also increased the final velocity of the particles because the gas molecules in air are sufficiently large to create drag on the flying particles. Accelerating the particles in a vacuum also necessitates that the target cells be under vacuum. Fortunately, mammalian cells can tolerate transient vacuum of 25 in. of mercury for long enough to allow transfection by gene guns like the PDS1000. Unfortunately, the same cannot be said for intact animals, who would likely explode if exposed to high vacuum. To protect animals from the required vacuum, the initial solution was to design an adaptor that could be placed in the vacuum chamber of the PDS1000 that would allow exposure of only a small area of skin or organ to the vacuum of the gun (14). The subsequent solution for the vacuum problem was the design of ''handheld'' gene guns (Fig. 3), in which the vacuum was internal to the gun and only a small area of tissue was exposed to vacuum via a small exit port (Fig. 2B). Intact tissues like the skin and liver are resilient to the application of vacuum levels of 30 to 20 in. of Hg, respectively, through the exit port of the gene gun (7). Even relatively fragile structures like the eye will tolerate vacuum levels of 5 in. Hg (17,18).

The second key feature that enabled the shockwave-generating gene guns was the use of kapton or mylar plastic disks as macrocarriers (7,8). These incredibly tough plastics are sufficiently durable to be struck by a supersonic shockwave and also hold up to the gas blast that creates the shockwave. The tough nature of these macrocarriers allowed the disks to perform several critical roles in the gene gun. Their primary function was to carry the microparticles as a ''flying disk'' macrocarrier that could be accelerated by the shockwave, fly to and impact with the stopping screen (Fig. 2A). When the macrocarrier impacts the stopping screen, the disk is stopped but the loosely held microparticles on the front of the disk do not stop and fly through the screen to impact the target cells below (Fig. 2A). The shockwave that is the driving force for particle acceleration travels at a speed greater than the speed of sound. The helium blast that produced the shockwave is chasing after the wave at the speed of sound. If this blast reaches the target cells or tissue, the target will be destroyed by the gas impact. To protect the target cells from this blast, the macrocarrier plays its final role upon impact with the stopping screen, where it seals the upper chamber with the helium away from the lower chamber containing the target cells. The helium blast chasing after the shockwave therefore bounces off the macrocarrier on the stopping screen and the excess pressure is absorbed by the vacuum pump (Fig. 2A). Thus, the flying disc macrocarrier not only carries the particles, but also shields the target from the gas blast that is the driving force of the gun.

Particle acceleration by entrainment differs substantially from shockwave-mediated gene guns. For entrainment, the

microparticles are literally ''blown'' out of a tube by high-pressure helium without any supersonic shockwave production in a manner analogous to blowing powder out of a straw (Fig. 2C). This entrainment approach originally described by Sanford did not become practical until gas valves could be used in the gene guns that could open and close rapidly enough to create a sharp gas accelerant burst. The entrainment approach to particle acceleration was first demonstrated by the group from Agracetus and later applied commercially in the Helios gene gun. Because a supersonic shockwave is not required for entrainment, much lower helium pressures (i.e., 200–400 psi) can be used for these types of gene guns. This approach also allows particle acceleration to occur in the absence of a vacuum. Although the gas pressure is lower, it is still sufficient to shred target tissues if not deflected. To deflect the helium, these guns typically use deflectors or a ''horn'' that dissipates the gas to the sides of the shot site using Bernouli's law (Fig. 2C). These guns are therefore easier to use than shockwave gene guns because entrained guns do not require vacuum or very high pressures of gas. They are, however, more prone to aerosolizing DNA or target materials into the environment because the helium surge is blown to the sides of the gun. One should be aware of the potential for aerosols with this type of gun, particularly when shooting potentially biohazardous materials on the particles or when addressing a biohazardous target (e.g., an infected cell culture, animal, or person). It is advisable to perform the shooting with this type of gun (or any gun) in a biosafety cabinet designed for personnel protection to avoid distribution of random agents into the air. The shockwave gene guns largely avoid aerosolization problems by applying shots under vacuum, but are more cumbersome to use than the Helios gene gun. Regardless of which gene gun is used, one should always consider the production of aerosols, how potential contaminants are released from the gun in the laboratory, and effective methods to decontaminate the gun after use.

E. Particle–Cell Interactions

Gene guns use kinetic energy to deliver nucleic acids, proteins, and chemicals directly into cells. As such, gene guns have the capacity to modify cells that are generally refractory to vectored gene delivery. For example, differentiated skeletal myotubes have historically been difficult to transfect with nonviral or viral vectors (19) due in part to lower receptor-mediated uptake by myotubes, as well as mismatch between the receptors of the cell and the ligands of the vectors (20). Although we could not transfect differentiated myotubes with liposomes or other vectors, these cells could be transfected by the gene gun (Fig. 5). Similarly, more complex targets such as whole mouse embryos can be transfected by the gene gun ex vivo with little damage to the organism (Fig. 6). Therefore, refractory cells can be addressed by the gun using kinetic energy to deliver the DNA directly into the cytoplasm of the cell, thereby avoiding the need to cooperatively bind a cell receptor, enter an endosome, and escape into the cytoplasm (Fig. 1).

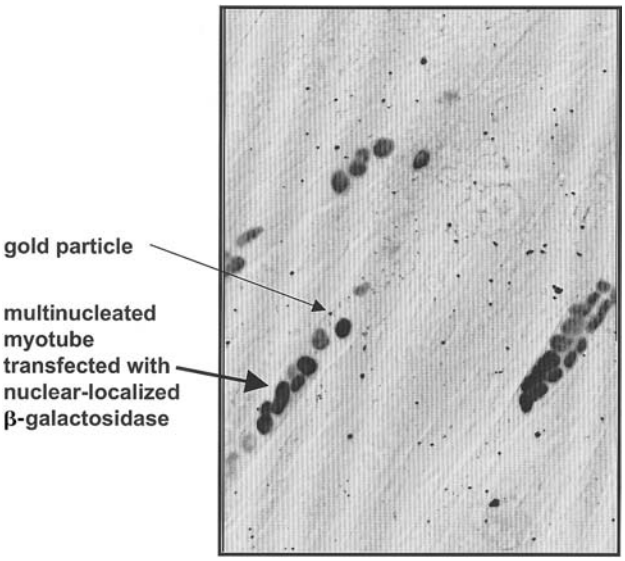

Figure 5 Gene gun-mediated transfection of terminally differentiated skeletal myotubes. Cells were cultured and differentiated and transfected with 1 to 3 μm gold particles coated with nuclear-localized β-galactosidase gene. One day later, the cells were fixed and stained with X-gal. The large dark circles are the β-galactosidase-positive nuclei of the multinucleated myotubes.

When tissues or cells in culture are examined to determine the location of the particles in the cells, they are in most cases found in the cytoplasm of the transfected cell, not in the nucleus (Fig. 5 and data not shown). Experiments using fluorescently labeled DNA have demonstrated that immediately after impact in the cytoplasm, DNA on the particles comes back

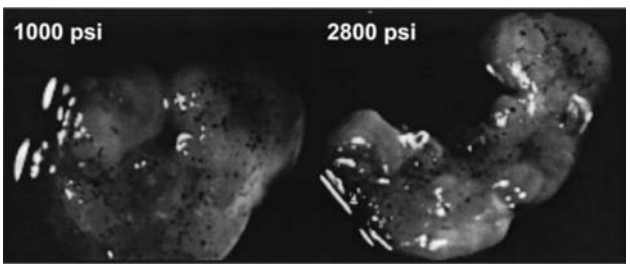

Figure 6 Gene gun-mediated transfection of living mouse embryos. Day 9.5 embryos were transfected in vitro with 1 to 3 μm gold particles coated with nuclear-localized β-galactosidase gene. One day later, the embryos were fixed and stained with X-gal. The dark dots under this low magnification are the β-galactosidase-positive cells.

into solution and rapidly ''streams'' into the nucleus of the cells (Stephen Johnston, personal communication). This observation has several implications. First, DNA is not delivered by direct particle delivery into the nucleus. Instead, the DNA is initially delivered into the cytoplasm where it engages endogenous host cell nuclear uptake machinery for translocation into the nucleus (Fig. 1B). This active uptake into the nucleus after gene gun delivery is quite different than that observed after microinjection of DNA into the cytoplasm were little uptake is observed (21–23). This difference could occur by a number of mechanisms. The first difference is related to how much DNA is introduced and how biologically relevant that amount of DNA is. For example, cytoplasmic microinjection delivers approximately 10 to 1000 femtograms of DNA in picoliter volumes (21–23). This amount of cytoplasmic DNA equals approximately 15,000 to 1,500,000 plasmids per cell. Given that each cell only has approximately 30 femtograms of cellular DNA, it is not surprising that the nuclear uptake machinery of the cell might be overwhelmed and disabled by the delivery of whole genome amounts (or more) of injected DNA. In contrast, the gene gun typically delivers 1 particle per cell where each particle carries approximately 100 copies of plasmid (0.006 femtograms of DNA). Therefore, under this condition of delivering a low number of plasmids to the cell in amounts that normally occur during transfection, DNA uptake by the nucleus is observed, whereas when massive amounts of DNA are delivered to the cytoplasm, the system is overwhelmed and nuclear uptake of DNA is inefficient. An alternate mechanism may involve a secondary effect of using kinetic energy to deliver particles and DNA to cells. In this mechanism, the velocity of the microparticles not only punches the particle through the plasma membrane, but also the impact of the particle with the cell may shatter this complex network of the cytoskeleton (Fig. 1B). This could disrupt the ''size exclusion'' or ''sieving'' effect of the cytoskeletal network that is speculated to impede movement of DNA through the cell (Fig. 1A). If this occurs, then the delivered DNA might be more easily translocated to the nuclear pore and into the nucleus than if DNA is simply injected into the cytoplasm. Another mechanism to explain more efficient nuclear uptake after gene gun delivery involves the use of the polyamines like spermidine to protect DNA when it is loaded onto the microparticles. In this case, the polyamine that is normally found in the nucleus bound to DNA may itself act as a nuclear localization signal to increase localization of the DNA to the nuclear pore and/or to increase uptake into the nucleus through the pore. For the gene gun, nuclear uptake does not appear to require nuclear membrane breakdown during mitosis (21) because one can effectively transfect a number of postmitotic cells, including the epidermis of the skin (14) and terminally differentiated myotubes (Fig. 5). These mechanisms (and others) may act alone or in concert to produce differences in the efficiency of DNA uptake into the nucleus by various gene delivery methods. Robust head-to-head comparisons using biologically relevant amounts of DNA are needed to extract the true biology at work.

F. Efficiency of Gene Gun Delivery

Although gene guns can address cells that are refractory to cooperative vectored gene delivery, the gene gun is limited in the total number of cells that are typically transfected in each shot. In most cases, the total number of cells addressed by this technology is typically 5% to 10% of cells within 10 to 20 cell layers of a target site (14). This limited capacity to transfect many cells at a time limits the gene therapy applications for which this technology will be effective. For example, the gene gun is clearly unsuited to gene therapy that requires that most if not all cells be modified (e.g., in Duchenne muscular dystrophy) because only a small fraction of target cells can be addressed. Likewise, the gene gun is unsuitable for therapeutic applications to tissues that are difficult to expose, such as the inner structures of the brain or the lumen of the lung. In contrast, the gene gun is suitable for addressing targets in culture, targets that are at the surface of the body [e.g., the skin (13,14), the eye (17,18)], or internal organs/tissues that can be exposed surgically [e.g., the liver (13,14), the muscle (13,24), the spleen (25)].

IV. APPLICATIONS OF GENE GUNS IN THE IMMUNE SYSTEM

Gene gun technologies are most potent when applied to easily exposed targets and when expressing gene products that have high-specific activity or whose biological effects engage systems that amplify the protein's effects. The most potent amplification system in the body is the immune system because this has evolved to detect a small number of infectious agents and amplify a response that may involve millions of cells and billions of effector molecules. Given this, some of the most potent application areas of the gene gun stimulate the immune system by delivering either antigens, cytokines, or both to the host organism. Current applications of the gene gun for immunological applications fall into the following categories:

1. Genetic immunization to provoke cellular and humoral immune responses by delivering antigen genes from pathogens or cancers to the immune system
2. Genetic immunization to divert problematic allergic or autoimmune responses by delivering antigens from the allergen or autoreactive immune cell or by delivery of cytokine genes to skew the T helper-type response
3. Cancer gene therapy to amplify weak responses to self- or mutant antigens present in tumors by delivering genes encoding immunostimulatory or immune presentation proteins

A. Genetic Immunization to Increase Immune Responses

The gene gun and naked DNA injection approaches for in vivo transfection with plasmids in mammals were developed

essentially in parallel in the 1980s. With the advent of these approaches, it became theoretically possible to use them to deliver antigen genes into the cells of a host to provoke immune responses. Genetic immunization is performed by introducing the gene(s) for protein antigens into the host animal rather than introducing the antigen itself. Once the plasmid is delivered into the host cell, the gene is expressed and produces the antigen intracellularly. The process therefore essentially uses the host animal itself as a bioreactor to generate its own vaccine antigens to drive both antibody and cellular immune responses.

1. History

The first published demonstration of this novel approach to immunization was by Stephen Johnston's group (26). In this first paper published in 1992, the ''wand'' gene gun (Fig. 3B) was used to deliver plasmids encoding human growth hormone and human α1-antitrypsin into the skin of mice. This in situ gene delivery allowed the host cells to produce these secreted proteins such that they were recognized by the immune system and provoked potent antibody responses against these foreign antigens. The authors coined the term ''genetic immunization'' to describe this process of using genes to immunize a host. Subsequent publications have called the technology ''DNA-based immunization,'' ''DNA vaccines,'' and ''polynucleotide vaccines.'' Recent terminology seems to have settled toward calling the process ''genetic immunization'' and the application a ''genetic vaccine,'' a ''DNA vaccine,'' or a ''gene-based vaccine.''

This first publication by Johnston's group was interestingly delayed by over a year and half by a number of short-sighted and erroneous reviews that produced comments such as ''*···this is cute at best···*'' and ''*···this will induce tolerance···*''. Even later reviews were skeptical about the utility of this technology with titles such as: ''*Genetic immunization—the biological equivalent of cold fusion*?'' (27). These reviewer comments were obviously off the mark in predicting the impact of the approach because Johnston's paper has been referenced more than 600 times since 1992, and there are now more than 350 publications in the PubMed database using the approach.

2. Antibody Production

Genetic immunization can elicit antibody responses against encoded antigens to make polyclonal and monoclonal antibodies in the laboratory (26,28). This application is particularly useful for raising antibodies against proteins that cannot be purified directly or that cannot be readily expressed in *Escherichia coli*, provided the gene for the protein is available. Antibody production is a practical nonvaccine application of the technology that is particularly useful in this genomic era of biology, when an investigator is more likely to possess the gene that encodes a protein before they will actually have the protein itself. As such, genetic immunization is a simple approach that allows the gene to be used to make antibody tools to analyze proteins and bridge the gap between genomics and proteomics. Given this, a number of companies now make their antibodies only by genetic immunization and a few offer this service commercially to investigators.

3. Vaccine Applications

Johnston's paper demonstrated that genetic immunization could be used to produce antibodies with a gene gun (26). Wolff's observation that muscle injection of naked DNA could transfect host cells (15) prompted several other investigators to explore whether plasmids could be used for vaccination against infectious agents, including influenza and human immunodeficiency virus 1 (HIV-1) (29–31). This approach was theoretically attractive for vaccine applications because these technologies allowed proteins to be produced intracellularly in living animals, such that they could be presented by major histocompatibility complex (MHC) class I molecules to stimulate T cell responses. Therefore, a cell would look infected to the immune response without actually having to be infected with a biohazardous agent. The feasibility of generating cellular immune responses by genetic immunization was first published in 1993 by Margaret Liu's group at Merck, where they demonstrated the production of cytotoxic T lymphocyte (CTL) responses against the nucleoprotein of influenza virus (29). In this same publication and in one published the same year by Harriet Robinson's group, both demonstrated that genetic immunization could be used to vaccinate animals and protect them from infection (31).

These observations provided excellent proof of principle for the application of genetic immunization for vaccine approaches driving both humoral and cellular immune responses. Prior to this, combined antibody and cellular responses could only generally be achieved using attenuated forms of the pathogen itself or by use of infectious viral vaccines that carry considerable biosafety baggage. In contrast, genetic immunization generated qualitatively similar immune responses as these whole pathogen or whole virus vaccines, but in the form of a single plasmid without any vector antigens. This meant that a genetic vaccine could provoke immune responses against its encoded genes without generating immune responses against itself, so multiple immunizations with the same vector were feasible. Likewise, since a genetic vaccine typically carries only 1 or a few genes from a pathogen, this type of vaccine could not cause the infection it is intended to prevent (32). This contrasts with potent whole pathogen vaccines such as that for poliovirus that can infrequently escape attenuation and actually cause the disease they should repel (33).

These benefits and others (Fig. 7) have led to the application of genetic vaccines against a growing list of infectious agents and against cancer for prophylactic and therapeutic immunization. The gene gun has been applied for vaccination against HIV, simian immunodeficiency virus (SIV), and feline immuno deficiency virus (FIV) (34–53), hepatitis (54–58), schistosoma (59), rabies and pseudorabies (60,61), encephalitis viruses (62–67), foot and mouth disease virus (68), rubella virus (69), infectious hematopoietic virus (70), papilloma virus (71,72), herpesviruses (73), pseudomonas aeruginosa

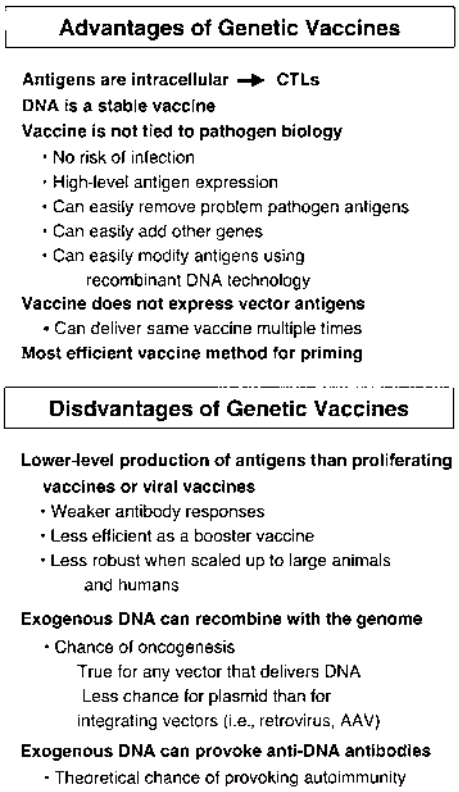

Figure 7 Advantages and disadvantages of genetic vaccines.

(74), chlamydia (75), and cancer (51,76–81). Beyond these demonstrations of genetic immunization for prophylactic vaccination, notable examples of gene gun applications of genetic vaccines are for biodefense [e.g., ebola virus (82), anthrax (83)], for emerging pathogens [e.g., ebola virus (82) and hanta virus (32)], and for unique vaccine applications [e.g., control of gingivitis (84)]. The gene gun has been applied for vaccination predominantly in mice, but also in rabbits (39,85), rats (86), chickens (31), cats (53), cattle (73,87), sheep (87), fish (70), nonhuman primates (35), and humans (58).

4. Genetic Immunization Allows Combinatorial Delivery of Antigen Genes for Multivalent Vaccines and Vaccine Discovery

In most applications against infectious agents, genetic immunization is performed to deliver a specific antigen gene from the pathogen to provoke protective cellular or humoral responses. Early genetic vaccines generally delivered single antigen genes to the host for immunization (29,30). Subsequent applications have included multiple genes to provoke multivalent immune responses (88). Another approach is to deliver whole genomes of pathogen genes by Expression Library Immunization (ELI) (50,89,90). The ELI approach can be applied to screen whole pathogen genomes to identify vaccine genes where none existed previously or can be used as an approach to delivery all or many antigens from a pathogen to provoke many immune responses simultaneously (50,89,90).

5. Reengineering Antigens

Unlike other vaccines, genetic vaccines are simple plasmids. Therefore, any recombinant technique can be applied to these vaccines to fundamentally manipulate how the vaccine is expressed and how it interacts with the immune system. Recombinant engineering of pathogen and nonpathogen plasmids has utility to increase antigen expression and boost the level of immune responses against these antigens. For example, antigens can be codon-optimized by rebuilding whole genes from oligonucleotides to replace poorly translated codons of pathogen genes with well-translated ones optimized for mammalian expression (91). Other approaches involve fusing poorly translated antigen genes to well-expressed mammalian proteins to increase translation *in cis*(92).

For pathogen vaccines, reengineering the antigen may be a necessity because many pathogens have evolved potent methods to hide not only themselves, but also their antigens from the immune system. As such, reengineering antigens in genetic vaccines provides an approach to break down immune evasion mechanisms that are built into the structure of pathogen proteins. Antigens can be reengineered to increase antibody responses by fusing them to secretory leaders or whole secreted proteins (89,93). $CD8^+$ CTL responses can be increased in genetic vaccines by fusing antigens to proteasome targeting proteins such as ubiquitin (89) or by fusing them to cytokine domains such as Flt-3 ligand (94). $CD4^+$ T helper responses can be increased by fusing antigens to lysosomal targeting proteins like LAMP-1 for intracellular targeting (95) and to proteins like the Fc domain of immunoglobulin for extracellular targeting to antigen-presenting cells (APCs) (96). Likewise, proteins can be delivered as a cocktail of plasmids, each expressing an overlapping fragment of the protein to break down secondary structures in the antigen that prevent processing by the proteasome and that hide subdominant epitopes (97). Fragmenting pathogen antigens also has the benefit of inactivating the function of proteins that may be frankly toxic or that inhibit immune presentation of pathogen antigens. By these methods, one can engineer vaccines that are markedly more potent than the pathogen or cancer itself.

6. Amplifying Genetic Vaccine Potency

Genetic vaccines have demonstrated robust protection against a wide variety of pathogens. In most cases, proof of principle has been demonstrated in mouse models where the amounts of DNA delivered could mediate high-level immune responses. Although many vaccines mediate protective levels of immune responses in large animals or humans, other genetic vaccines have failed this scale-up when translated into larger animals (36,37). This has been a particular problem for human immunodeficiency virus (HIV-1), simian immunodeficiency virus (SIV), and shiv chimeric (SHIV) vaccines aimed at testing in nonhuman primates, where initial genetic vaccines failed to mediate protection (36) or mediated only partial protection (37). These weaker responses in larger animals are likely due

to the fact that genetic vaccines generally produce nanogram to microgram amounts of antigen, levels that may be inadequate to effectively stimulate potent immune responses in a large mammal. A number of approaches are being tested to amplify genetic vaccine immune response for human applications, including (1) codon optimization, (2) coadministration of cytokine genes, and (3) use of heterologous vaccines to boost genetic vaccine priming.

7. Codon Optimization

Early work using genetic immunization with HIV-1 envelope plasmids generally raised weak antibody responses (98–100). This problem appears to be due in part to the poor codonbias of HIV genes that makes their expression in mammalian drastically reduced compared with other genes (91). The level of expression of envelope can be increased more that 100-fold by codonoptimization in which codons that are poorly represented by mammalian tRNAs can be replaced by codons from highly expressed mammalian genes. This increased expression by codonoptimization produces recombinant HIV genes that mediate substantially better immune responses after genetic immunization (101). Given this, most current genetic vaccine trials in primates or humans now use antigen genes that have been completely reengineered by codonoptimization strategies (102).

8. Coadministration of Cytokine Genes

In some cases, cytokine-expressing plasmids have been added to the plasmid mixture as genetic adjuvants to augment immune responses against pathogens (103–105). However, most genetic vaccine approaches against pathogens are directed at delivering antigen genes rather than cytokine genes. This contrasts with most gene gun applications for cancer, where it is more typical to deliver cytokines into tumors or tumor cells (106) because, in many cases, cancer antigens and their genes are unknown. Combining genetic vaccines with cytokine plasmids as genetic adjuvants is a robust method to increase immune responses (104,107,108). This approach has recently been quite effective at amplifying not only CTL responses, but also increasing control of pathogenic viruses in rhesus macaques (102). In this case, a relatively simple genetic vaccine expressing codon-optimized SIV gag and HIV env was used to immunize rhesus macaques by intramuscular (i.m.) injection. This work demonstrated control of viremia in the macaques only when IL-2-Ig plasmid (a more stable IL-2 protein) was inoculated with the genetic vaccines (102). This highlights the application of cytokine genetic adjuvant to rescue genetic vaccine responses and also highlights the need to provide robust T helper cell support for vaccines aimed at driving CD8 T cell responses.

9. Use of Heterologous Vaccines for Boosting

An alternate approach to amplify immune responses has been to boost genetic vaccines with heterologous vaccines such as recombinant protein vaccines or recombinant viruses (109,110). The improved responses observed with protein or viral vector boosting are likely related to the larger amounts of antigen that are delivered by these methods. Although these protein or viral vaccines are useful for boosting, in most cases, they are not as potent as genetic vaccines for immune priming, perhaps by the ability of DNA vaccines to directly transfect dendritic cells (111) or to cross-prime antigens to dendritic cells (112). Genetic vaccines are also useful for priming, because they do not encode vector-specific antigens. In contrast, viral vectors such as vaccinia or adenovirus generate immune responses against the intended vaccine antigen and also against proteins of the viral carrier. Therefore, when viral vectors are used, they generate immune responses against themselves that preclude their effective readminstration. In contrast, genetic vaccines express only the vaccine antigen. Thus, they impart no immunological memory against the vector itself and can be readministered multiple times or used to prime antigen-specific responses for later amplification by viral vectors with more robust antigen production. Given these observations, a number of protocols intended for humans involve DNA priming with a heterologous vaccine for boosting. One good example of this is a combined DNA and modified vaccinia ankara (MVA) poxvirus vaccine that is now being developed for a phase III clinical trial in Africa as a candidate acquired immune deficiency syndrome (AIDS) vaccine (113). Similarly, Merck is pursuing a DNA prime and adenoviral vector boost strategy to amplify responses (reviewed in (61)).

10. Genetic Immunization of Humans

Although the gene gun has been applied to many types of animals, only 1 pathogen-directed application has been reported in the literature in humans. In this trial, Powderject Vaccines is testing a hepatitis B virus genetic vaccine using the gene gun to immunize healthy volunteers (58). Three groups of 4 people were immunized epidermally by gene gun with 1, 2, or 4 μg of plasmid-encoding hepatitis B virus surface antigen. The immunization was well tolerated and provoked CD8 T cell responses as well as protective levels of antibodies in all volunteers. The amount of DNA used in this gene gun trial contrasts with other trials using naked DNA injection that use 0.1 to 1.8 milligrams of DNA (114–119).

It should be noted that China is well ahead of the United States in transferring genetic vaccines into humans as demonstrated by the fact that, since 1997, the primary clinical vaccine against hepatitis B in China is a genetic vaccine (120). Although not a gene gun approach, this large scale application of genetic vaccines in China is a fundamental milestone in this vaccine technology.

11. Gene Guns Versus Naked DNA Injection for Genetic Vaccines

Investigators in genetic immunization tend to fall into two camps: those that use gene guns and those that use naked DNA injection. Prior to commercialization of the Helios, most investigators could not obtain a gene gun unless they built one themselves or entered into a research collaboration with companies making guns. Even now when one can buy a gene gun commercially, the cost of the instrument runs in excess of $15,000 for academics, making it prohibitive to many investigators. Given these parameters, the vast majority of ge-

netic vaccine research has been performed using a syringe and needle to inject large amounts of plasmids as naked DNA in saline. Based on Wolff's results (15), most work has involved naked DNA injection into skeletal muscle. Raz's group subsequently demonstrated that intradermal injection could also be used for genetic immunization (121). At least in mice, the site of intradermal injection has marked effects on the efficiency of transfection, where injection into the rigid structure of the tail can generate 10- to 100-fold higher transfection than i.m. injection or injection at other dermal sites (2). This may be due to the ability to produce higher pressures upon injection into the tough tail versus generating lower pressures in other sites that can expand upon injection. Indeed, the fact that injections in small animals work substantially better than in large primates may be due to the fact that injections into mouse muscle deliver 25 to 50 μL of volume into a tissue that may itself only normally be 50 to 500 μL in volume. In contrast, injections into primates and humans are never delivered with same relative volumes as in mice. Given that high-pressure injection into a number of tissues including muscle increases transfection efficiency (122,123), it is likely that the poor scale-up of naked DNA injection large animals and humans is in part due to the use of too small of volumes into the muscle and dermis.

In many applications, the gene gun and naked DNA injection yield comparable immune responses when both are applied under optimal conditions. However, the fundamental mechanisms by which cells are transfected by the 2 approaches are quite different and can yield qualitatively different immune responses.

12. Gene Guns Transfect Dendritic Cells and Non-APCs, Naked DNA Injection Transfects Non-APCs

The gene gun is typically applied for genetic immunzation by directly transfecting epidermal cells of the skin. As such, the gene gun can not only transfect nonantigen-presenting keratinocytes, but the gun also transfects Langerhans dendritic cells directly in vivo (111). In contrast, naked DNA injection predominantly transfects non-APCs when applied in the skeletal muscle. Therefore, in i.m. injection, the vast majority of antigen presentation to T cells occurs by transfer of antigens from the transfected muscle cells to host APCs for cross-presentation (124). In contrast, the gene gun may drive immune presentation either by cross-presentation or by direct transfection of dendritic cells, allowing these transfected APCs to express and present antigens themselves. Although some work delivering antigen plasmids under the control of tissue-specific promoters has suggested that gene gun-mediated immune responses occur only by cross-presentation (125), the promoters used were somewhat mismatched for the biology of epidermal cells and were not directly validated by in vivo reporter activity. Other work has compared the efficiency of gene gun modified Langerhans cells that were transfected either directly in vivo or in vitro and then reintroduced into the animals (126). In this case, the investigators found that transfection of as few as 500 dendritic cells in vitro in the absence of non-APC transfection-generated immune responses comparable to that by direct gene gun delivery. Although direct transfection of dendritic cells in vivo by the gene gun appears promising, it is conceivable that this transfection may actually render the cells less efficient at presentation, or they may only inefficiently present their antigens expressed in an autocrine fashion. Further work is required to determine which transfected cells is driving immune responses after gene gun transfection.

13. Gene Gun Delivery Is More Efficient and Consistent Than Naked DNA Injection

The gene gun is inherently more consistent shot to shot than i.m. injection (108). Increasing the amount of DNA in an i.m. injection increases the ''hit'' rate, but does not obviate animals that fail to respond (Fig. 8). To reduce the likelihood that an animal will be ''missed'' by i.m. injection in a given immunization round, it is advisable to inject each animal in four separate sites. To achieve the same level of antigen production, one typically needs to deliver 50 to 100 times as much plasmid by naked DNA injection than by gene gun (31,108). Typical delivery amounts for the gene gun are 1 to 2.5 μg per shot. For i.m. naked DNA injection, they are typically 50 to 100 μg per shot in a mouse. This difference in efficiency is likely related to the ability of the gun to deliver plasmids directly into the cytoplasm of cells, whereas syringe injection delivers DNA extracellularly where more than 99% of the injected DNA is rapidly degraded into nonexpressible DNA fragments by the action of extracellular nucleases (2).

14. T Helper Cell Bias for Gene Guns and Naked DNA Injection

Depending on the pathogen, protection can be mediated antibody responses, cellular responses, or a combination of both. For some infectious agents, antibodies are able to neutralize incoming pathogen. For other pathogens, $CD8^+$ T cell responses are advantageous because these can kill or inactivate infected or neoplastic cells. Fortunately for viral and cancer vaccines, the plasmid DNA used for genetic vaccines is inherently biased toward generating $CD8^+$ T cell by virtue of the fact that it is quite potent at biasing T helper responses toward a T_{H1} phenotype. This bias occurs because the bacterial plasmid DNA bears unmethylated CpG motifs that stimulate IFN-γ and IL-12 production from natural killer (NK) cells and macrophages (127). This production of cytokines biases immune responses toward the T_{H1} phenotype against any antigen present after injection, whether it is encoded by the DNA itself or delivered as a protein (128).

Because naked DNA injection delivers 50 to 100 times as much DNA as the gene gun, i.m. and intradermal (i.d.) injection has a stronger T_{H1} bias than the gene gun [(24) and Fig. 8]. Both methods of gene delivery are able to drive T_{H1} responses at the early phases of responses as evidenced by interferon (IFN)-γ production by T helper cells, but the gene gun appears to have increased T_{H2} character (at least for some antigens) later in the T helper responses, as evidenced by increases in interleukin (IL)-4 production after IFN-γ re-

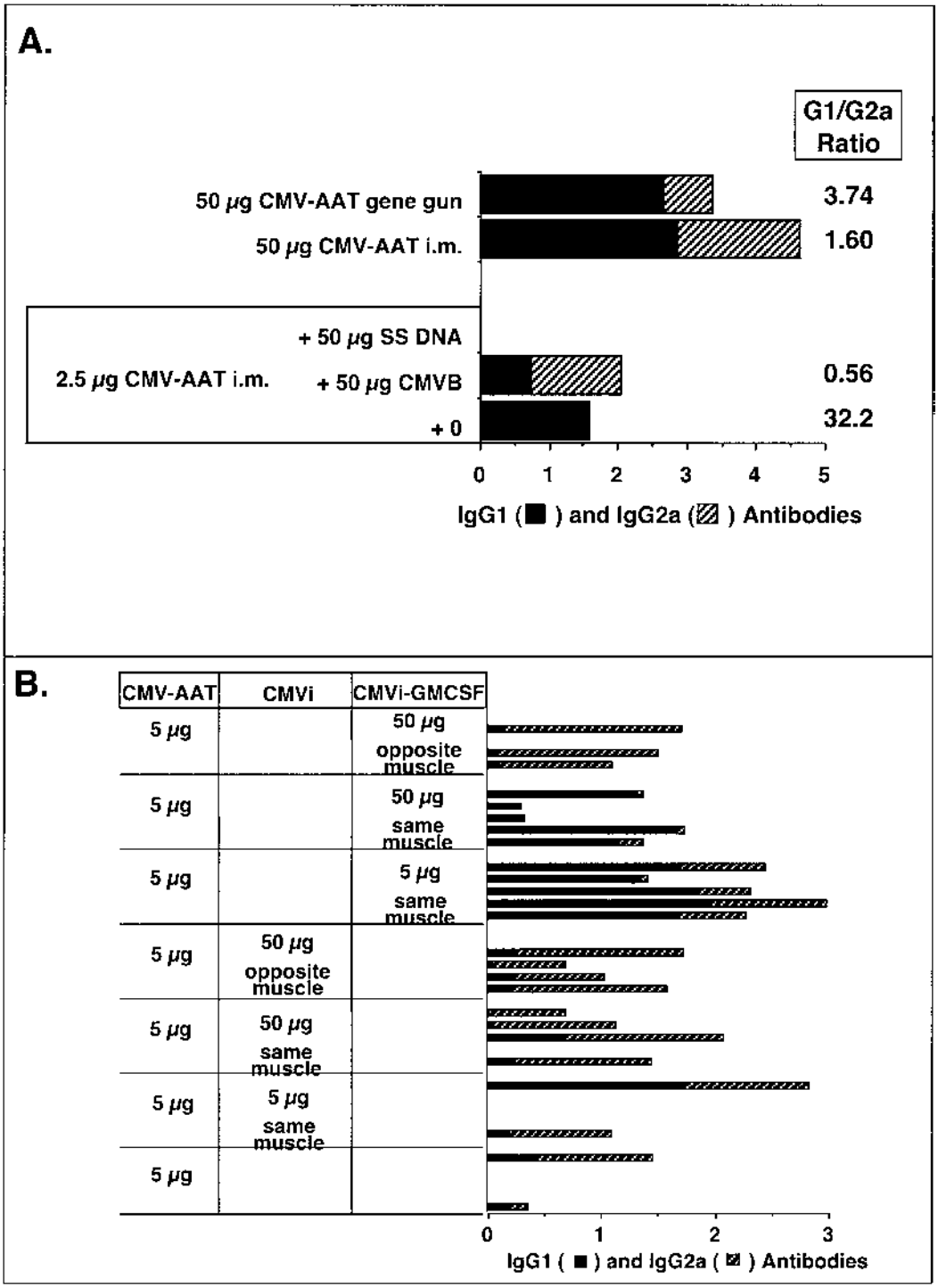

Figure 8 T_{H1} skewing by excess plasmid DNA after gene gun and i.m. injection. BALB/c mice were immunized a single time as indicated, and their antibody responses were evaluated by isotype-specific ELISA against α1-antitrypsin (AAT). (A) Comparison of the effects of large amounts of antigen-coding plasmid (CMV-AAT), noncoding plasmid (CMVB), and eukaryotic DNA (SS DNA) on antibody levels and isotype. (B) Comparison of the effects of noncoding excess DNA and GM-CSF expressing plasmid on antibody boosting and isotype when delivered into the same site as the antigen plasmid or the contralateral muscle.

sponses wane (129). Because of this, the gene gun fairs worse in controlling some pathogens that are highly dependent on potent $CD8^+$ T cell responses for protection (130). Conversely, the T_{H2} bias is the likely explanation for why the gene gun is generally more robust at generating antibody responses than i.m. injection in mice (131) and in primates (132). Therefore, the gun has better utility for protection requiring neutralizing antibodies [e.g., hepatitis B (58)]

Although many investigators call i.m. injection ''T_{H1}'' and the gene gun ''T_{H2}'', this is inaccurate. It is more accurate to say that both can produce mixed T_{H1}/T_{H2} responses and that i.m. injection skews responses toward T_{H1}, whereas the gene gun skews responses toward T_{H2}. The T_{H2} bias of the gun is not absolute because the gene gun can provoke quite potent T_{H1}-driven CD8+ T cell responses with nanogram amounts of plasmid (131) and even after single immunization (50). Further, the T_{H2} bias of the gene gun can be converted to more of a T_{H1} responses by coimmunization with cytokines like IFN-α or IL-12 (133).

Each antigen has its own T_{H1} or T_{H2} bias and the different delivery methods retain the inherent bias or skew it in one direction or the other. For example, protein immunization with human α1-antitrypsin (AAT) in mice produces IgG_1 antibodies. If a 2.5 μg of a plasmid-encoding AAT is delivered by either gene gun or i.m. injection, antibody responses are still predominantly IgG_1 (Fig. 8 and data not shown). T_{H1} skewing (as evidenced by increased IgG_{2a} antibodies) only occurs when more bacterial DNA is added in the i.m. injection (either more AAT plasmid or a nonexpressing plasmid) (Fig. 8A). A similar mixed response involving combined IgG_1/IgG_{2a} antibodies is observed if 1 gene guns 50 μg of DNA. If 2.5 μg AAT plasmid plus 47.5 μg more AAT plasmid is introduced by i.m. injection, the response is still predominantly IgG_1. In contrast, if one adds 2.5 μg AAT plasmid plus 47.5 μg of a nonexpressing plasmid (supplies cytosine-guanosine dinucleotides (CpGs), but not more antigen), then stronger skewing toward IgG_{2a} responses is observed after i.m. injection (Fig. 8). Therefore, for AAT, the default antibody response is IgG_1. Adding more CpGs by gun or i.m. injection skews this toward a T_{H1} response as evidenced by increased (but not only) IgG_{2a} antibodies.

The CpG effects by plasmid DNA appears to be a systemic one given that increased responses can be observed, whether the large amounts of plasmid is injected into the same site as the antigen-expression plasmid or into a contralateral muscle (Fig. 8B). Increased levels of response as well as increased numbers of animals that respond are observed, whether large amounts of plasmid DNA is injected in the same site or an alternate site to where the antigen plasmid is injected. This site effect is quite different than that observed when using cytokine genes as genetic adjuvants. For example, low-level antibody responses after i.m. injection of suboptimal amounts of α1-antitrypsin (AAT) plasmid can be amplified if a granulocyte-macrophage colony stimulating factor (GM-CSF) plasmid is coinjected in the same site, but not if the cytokine plasmid is injected in another muscle (Fig. 8B). Note also, that unlike the CpG effect, the GM-CSF antibody boost retains the normal T_H-bias of AAT by producing predominantly IgG_1 antibodies. Interestingly, if GM-CSF plasmid is delivered into a nonmuscle site (e.g., the skin), this can actually antagonize the immune response (unpublished observations), suggesting the cytokine may attract APCs away from the site of antigen production to the wrong location.

In summary, the gene gun tends to bias responses toward a T_{H2} phenotype. Naked DNA injection biases responses toward the T_{H1} phenotype due to delivery of large amounts of unmethylated CpG motifs. Both approaches have the capacity to drive mixed T_{H1} and T_{H2} responses, and both can be skewed toward one response or the other by delivery of cytokine plasmids. The T_H bias of the antigen is a primary factor in the character of responses. One should be aware that differences in responses are usually highly antigen and model specific, so the approaches with generally need to be compared for each investigator's own system.

B. Genetic Immunization to Reduce or Skew Immune Responses

Allergy and autoimmune diseases represent immunological diseases in which aberrant immune responses occur against exogenous allergens or against self-antigens, respectively. Genetic immunization has been applied in these situations to inactivate or skew the existing immune response to one that does not support cause the disease. Genetic immunization prevents allergic responses when the allergen antigen gene is delivered with CpG-rich DNA to skew the T_{H2}-driven allergic response to a nonproblematic T_{H1} response. This approach has been applied successfully to prevent allergic responses against peanut (134), pollen (135), and milk antigens (136). For diseases like autoimmune diabetes, there are autoimmune T cell responses against antigens associated with pancreatic β cells. In NOD mice, the diabetes is mediated by responses against glutamic acid decarboxylase. Genetic immunization with a plasmid-encoding glutamic acid decarboxylase was able to prevent spontaneous diabetes in the NOD mice, demonstrating that the skewed responses could disrupt disease progression (137). An alternate approach is to generate immune responses against the antigen receptors of T cells that target self-antigens. Proof of principle for this approach was demonstrated for autoimmune encephalitis by expressing the genes encoding T cell receptor specific to the self-antigen myelin basic protein (138). This report was notable because the vaccine approach not only ablated the autoimmune disease prophylactically, but was also able to attenuate established autoimmunity. These reports on both allergy and autoimmune applications hold great promise for the application of genetic immunization for not only inducing new immune responses, but also ablating problematic immune responses.

C. Gene Gun Applications for Cancer

The use of gene guns and genetic immunization for cancer is usually quite different than for vaccination against pathogens. First, for most pathogens, candidate vaccine antigens are already known and can be applied in the genetic vaccine. For

those pathogens that lack candidate genes, ELI can be applied to screen the whole genome to find these candidates (89). In contrast, the proteins that could act as protective antigens against cancer cells are (1) usually unknown, (2) are usually specific to 1 type of cancer, and (3) may in some cases be specific to the individual patient. For those few cancers associated with or caused by infectious agents, one can vaccinate with genes from the pathogen that are associated with the tumor cell. Good examples of this are the use of the gene gun to vaccinate against papillomavirus antigens (139,140). Whole genome searches for antigens by ELI and other techniques is a staggering task for cancer as compared with viral or bacterial pathogens because the mammalian genome is 1000- to 10,000-fold larger than the genomes of most pathogens. To combat this size issue, most investigators use cDNA libraries, rather than unbiased genome libraries, to reduce the complexity of their search. Although feasible, screening cDNA libraries for cancer antigens introduces a potent bias in the antigens that are searched because representation in the library is directly proportional to the expression level of the message in the cancer cells. Despite these considerable difficulties, investigators have identified several promising cancer antigens. Given this, the gene gun has been applied to deliver melanoma antigens gp100 (80) and tyrosinase-related protein 2 (81), preprocalcitonin has been applied for medullary thyroid carcinoma (77), and alpha-fetoprotein has been applied for genetic vaccination against hepatocellular carcinoma (76).

The vast majority of gene gun applications for cancer are directed at generating cancer vaccines. In this application, the gun is used to transfect cancer cells either in vitro or in vivo, with plasmids encoding immunostimulatory proteins to amplify weak anticancer responses. By this approach, the cancer cell provides the antigens and the gene gun provides gene products to increase immune reactions against these antigens. One of the earliest approaches using gene guns for this application was by Kam Hui's group in Singapore, where they engineered their own gene gun and used this to transfect cancer cells with the genes for allogeneic MHC I proteins (78,141). Other applications have delivered genes encoding HLA-A, HLA-DR, and B7 costimulatory molecules (51). In most applications, the gene gun has been used to deliver cytokine genes to explanted tumors or tumor cell lines ex vivo (51,142–144). The advantage of the gene gun in these applications is that it mediated gene delivery without biohazardous viruses and without the need for cancer cells to be cycling or cultured for long periods of time. Alternate applications have delivered cytokines to the skin immediately above subcutaneous tumors (145–150), taking advantage of the ease in addressing the epidermis with the gun. Cytokines, including IL-2, IL-12, pro-IL-18, IFN-α, GM-CSF, and tumor necrosis factor (TNF)-α, have been delivered either alone or in combination to modulate cellular immune responses against the cancer cells. In the case of IL-12, direct transfection of the skin above intradermal sarcomas in mice mediated comparable protection to treament with recombinant IL-12 protein (151). However, an advantage associated with the localized delivery and production of IL-12 by the gene gun is that this avoided many of the toxic side effects associated with systemic IL-12 therapy (151). Further, transfection with cytokine genes by gene gun is substantially less expensive than systemic delivery of recombinant cytokine proteins.

Although the vast majority of gene gun testing for cancer has occurred in mouse models, 3 trials have been performed or are underway in humans. In 1 trial, melanoma and sarcoma explants from patients are to be transfected by the gene gun with a plasmid-encoding human GM-CSF and the cells were injected intradermally back into the patients as an autologous cancer vaccine (79). In 2 other trials, IL-7 or IL-12 plasmids were delivered by gene gun into melanoma cells and injected subcutaneously back into patients (152,153). Patients immunized with autologous IL-7-modified cells demonstrated little toxicity, increased CTL levels against the tumor, and 2 of the 6 patient demonstrated minor clinical responses (152). In the IL-12 trial, mild fever was the only side effect of the therapy and increases were observed in cytotoxic T cells (153). One of the 6 patients had a minor clinical response that was associated with infiltration of CD4 and CD8 T cells into metastatic sites distant from the vaccination site. From these phase I trials, the gene gun holds some promise for human tumor applications, particularly when it is applied to deliver known tumor antigens or when it is used to deliver cytokine genes for local production to augment immune responses against unknown tumor antigens.

V. GENE GUN-MEDIATED GENE THERAPY IN OTHER SYSTEMS

A. Gene Gun Applications for Wounds and Burns

The gene gun is most useful in applications on exposed tissues. Given this, a number of investigators are testing gene guns for their ability to increase repair of wounds or burns as well as to reduce pain signaling at sites of tissue injury [reviewed in (154)]. Examples of repair applications include delivery of keratinocyte growth factor (155), platelet-derived growth factor (156), and epidermal growth factor receptor (157) to speed wound healing. Examples of approaches to limit pain are delivery of semaphorin A (18) and delivery of the gene for pro-opiomelanocortin (158).

B. Gene Gun Applications in the Eye

Given that the eye is exposed on the surface of the body, this tissue is a natural target for gene gun applications. The first demonstration of transfection by the gene gun was in the delivery of plasmids encoding the green fluorescent protein to corneal epithelial cells (17). In this work in rabbits, it was demonstrated that gene gun particle penetration could be readily titrated by changing the helium pressure driving particle acceleration and that little corneal damage was observed after particle bombardment. This work was followed by a true gene therapy approach where the gene for semaphorin A was delivered into the eye to modulate neuronal growth and architecture

(18). This was a good example of a gene therapy approach that avoids immunogenicity problems because (1) the transgene product is already present in the host, but is being increased locally; and (2) the gene gun delivers simple plasmids encoding only the therapeutic transgene protein and no vector-specific antigens. This contrasts with viral methods of gene delivery to the eye that can provoke strong immune responses even in this ''immunopriviledged'' site (159).

Subsequent approaches using the gene gun in the eye have delivered fluorescent tracer chemicals and the genes for fluorescent proteins to the eye to characterize morphology (120,160,161) and to characterize eye-specific regulatory elements in enhancer/promoters of genes (162). Another interesting gene therapy application of the gene gun in the eye was to deliver genes encoding immunosuppressive proteins like IL-4 and CTLA4 into the cornea after an allotypic corneal transplant (163). In this case, gene gun delivery was able to prolong allogeneic graft survival in mice. This interesting application, like that for semaphorin A, delivers an endogenous gene product by simple plasmid to avoid immune responses against the transgene proteins. This work also provides proof of principle for the use of the gene gun to genetically modify a variety of tissues to increase transplant survival.

One limitation of the gene gun is that transfection generally occurs within 10 to 20 cells of the surface that is bombarded. As such, the gene gun will likely have utility in applications to genetically modify the surface of the eye, including the corneal epithelium and the conjuctiva. In contrast, applications in the retina or other buried structures of the eye will likely be out of reach of this technology.

C. Neuronal Gene Gun Applications

Most gene gun applications in neuronal systems have been performed for basic research studies to characterize the biology of these cells. The gun provides the unique ability to deliver substances directly into not only neuronal cells in culture (164,165), but also into whole brain slices (120,166). The gene gun has been used to deliver not only DNA, but also fluorescent tracer molecules to track neuronal phenotype and survival (165). Direct gene gun delivery into the brain in vivo has been reported for transfection of the lamprey brain neurons and glia in the exposed floor of the fourth ventricle (167), and for direct transfection of the exposed brain in live mice (168). An alternate in vivo approach of gene guns has been to manipulate neuron behavior, but not to directly transfect the neurons themselves. In this case, the gene for semaphorin A was delivered into corneal epidermal cells of rabbits (18). In this model, local production of semaphorin A after gene gun transfection effectively repelled established and growing A-delta and C-fiber trigeminal sensory afferents. This application provided proof of principle for gene therapy applications to repel pain-sensing neurons from locals to attenuate chronic pain or prevent problematic pain responses in surgical fields.

D. Gene Gun Applications in Other Organs

Although the gene gun is most easily applied to exposed tissues such as the skin or the eye, the original demonstrations of gene gun transfections in animals showed that internal tissues could be surgically exposed to allow gene gun delivery (13,14). Therefore, any tissue that can be surgically exposed can also be transfected by the gene gun. Applications to date on internal organs include the liver (13,14), the muscle (13,24), the spleen (25), the bladder (169), and the heart (170,171). In many cases, the gene gun has been used to probe the biology of cells or genes in these organs. In particular, the gene gun has been used to deliver genes to analyze the specificity and level of gene expression by different enhancer/promoter constructs into the liver (172) and into cardiomyocytes (173). In other cases, preclinical approaches are being tested to deliver antigen genes directly into the liver to prevent the liver stage of malaria (174). Future applications of the gene gun in deep tissues may be focused not only on understanding the biology of genes and tissues, but also for direct gene therapy. One interesting future application in organs will be to test whether the gene gun can effectively protect transplanted tissue from immune rejection. The work in corneal transplantation protection in the eye (163) provides good proof of principle for the use of the gene gun to modify tissues after removal without the need for immunogenic viral vectors or the need for prolonged culture ex vivo.

VI. OTHER PRACTICAL APPLICATIONS OF GENE GUNS

Beyond true gene therapy applications, the gene gun has a number of practical uses in the laboratory for basic science research or to enable other therapeutics. First, the gene gun has utility as a simple method to deliver enhancer/promoter constructs into cells to analyze gene regulation. The gene gun can also deliver gene-encoding proteins that interact with other proteins to assess pathway function. The gene gun is unique in these applications in that it can easily transfect a number of tissues in living embryos (Fig. 6) or living animals [(162,175) and Fig. 4]. Further, because the gun delivers plasmids, investigators do not have to go through the laborious process of engineering a new viral vector for each new enhancer, new intron, new poly A signal, or each new gene to be tested (Fig. 9). Because approximately 100 plasmids are complexed on each particle, each transfected cell receives multiple copies of plasmid. Because of this, the gene gun is efficient cotransfection of cells (6,176). This allows more efficient control of multigene systems, such as drug-regulated expression systems, than mediated by other vectors when applied in vivo. Finally, when applied for genetic immunization, the gene gun is a potent method to generate polyclonal and monoclonal antibodies (28) for laboratory and clinical applications.

VII. SUMMARY

Gene guns are a unique method of gene delivery that employs kinetic energy to overcome transfection bottlenecks to deliver chemicals, proteins, mRNA, or genes directly into cells on

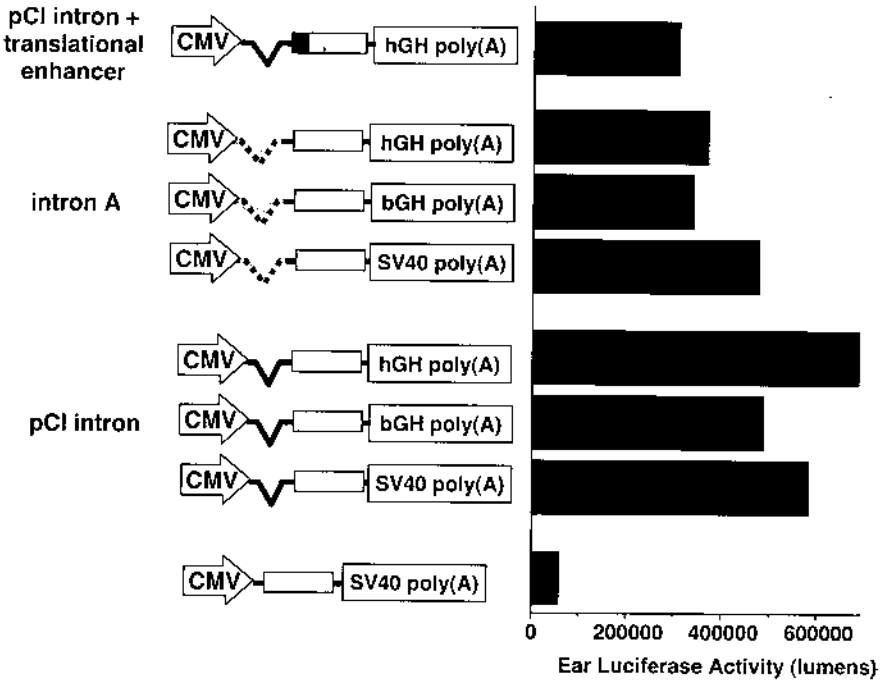

Figure 9 Rapid in vivo expression screening of different introns and poly adenylation sequences by gene gun delivery into the skin. Plasmids bearing the indicated sequences driving luciferase expression were delivered into the skin of the ears of BALB/c amice by gene gun. Expression comparison was performed the next day by luciferase assay on the shot skin.

microparticle ''bullets.'' Gene guns can transfect many tissues that are refractory to vectored gene delivery, and are also quite efficient at direct delivery of plasmids into living animals and humans. This approach allows simple plasmids to be delivered into the host that encode only the gene of interest. This avoids problematic immune responses against vector antigens that can prevent multiple administration. Although gene guns can modify both dividing and nondividing tissues in vitro and in vivo, they do not modify a massive number or percent of cells in a given transfection event. Therefore, gene guns are best applied to systems that amplify the effects of a small amount of transgene product. Gene guns therefore have great utility in the delivery of antigen or cytokine genes into the immune system to stimulate or block immune responses. Gene guns also have utility in surgical settings to repulse neurons to reduce pain and for the delivery of transgene proteins to enhance wound and burn repair.

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16

Optimization of Nonviral Gene Therapeutics

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I. INTRODUCTION

Many investigators are focused on the production of effective nonviral gene therapeutics and on creating improved delivery systems that mix viral and nonviral vectors. Use of improved liposome formulations for delivery in vivo is valuable for gene therapy and would avoid several problems associated with viral delivery. Delivery of nucleic acids using liposomes is promising as a safe and nonimmunogenic approach to gene therapy. Furthermore, gene therapeutics composed of artificial reagents can be standardized and regulated as drugs rather than as biologics. Cationic lipids have been used for efficient delivery of nucleic acids to cells in tissue culture for several years (1,2). Much effort has also been directed toward developing cationic liposomes for efficient delivery of nucleic acids in animals and in humans (3–12). Most frequently, the formulations that are best to use for transfection of a broad range of cell types in culture are not optimal for achieving efficacy in small and large animal disease models.

Much effort has been devoted to the development of nonviral delivery vehicles due to the numerous disadvantages of viral vectors that have been used for gene therapy. Following viral delivery in vivo, (1) immune responses are generated to expressed viral proteins that subsequently kill the target cells required to produce the therapeutic gene product, (2) an innate humoral immune response can be produced to certain viral vectors due to previous exposure to the naturally occurring virus, (3) random integration of some viral vectors into the host chromosome could occur and cause activation of proto-oncogenes resulting in tumor formation, (4) clearance of viral vectors delivered systemically by complement activation can occur, (5) viral vectors can be inactivated upon readministration by the humoral immune response, and (6) potential for recombination of the viral vector with DNA sequences in the host chromosome that generates a replication-competent infectious virus also exists. Specific delivery of viral vectors to target cells can be difficult because 2 distinct steps in engineering viral envelopes or capsids must be achieved. First, the virus envelope or capsid must be changed to inactivate the natural tropism of the virus to enter specific cell types. Then sequences must be introduced that allow the new viral vector to bind and internalize through a different cell surface receptor. Other disadvantages of viral vectors include the inability to administer certain viral vectors more than once, the high costs for producing large amounts of high-titer viral stocks for use in the clinic, and the limited size of the nucleic acid that can be packaged and used for viral gene therapy. Attempts are being made to overcome the immune responses produced by viral vectors after administration in immune-competent animals and in humans, such as the use of gutted adenoviral vectors or encapsulation of viral vectors in liposomes (13). However, complete elimination of all immune responses to viral vectors may be impossible.

Use of liposomes for gene therapy provides several advantages. A major advantage is the lack of immunogenicity after in vivo administration, including systemic injections. Therefore, the nucleic acid–liposome complexes can be readministered without harm to the patient and without compromising the efficacy of the nonviral gene therapeutic. Improved formulations of nucleic acid–liposome complexes can also evade complement inactivation after in vivo administration. Nucleic acids of unlimited size can be delivered ranging from single nucleotides to large mammalian artificial chromosomes. Furthermore, different types of nucleic acids can be delivered, including plasmid DNA, RNA, oligonucleotides, DNA-RNA chimeras, synthetic ribozymes, antisense molecules, RNAi, viral nucleic acids, and others. Certain cationic formulations can also encapsulate and deliver viruses (13), proteins or par-

tial proteins with a low isoelectric point (pI), and mixtures of nucleic acids and proteins of any pI. Creation of nonviral vectors for targeted delivery to specific cell types, organs, or tissues is relatively simple. Targeted delivery involves elimination of nonspecific charge interactions with nontarget cells, as well as addition of ligands for binding and internalization through target cell surface receptors. Other advantages of nonviral vectors include the low cost and relative ease in producing nucleic acid–liposome complexes in large scale for use in the clinic. In addition, greater safety for patients is provided using nonviral delivery vehicles due to few or no viral sequences present in the nucleic acids used for delivery, thereby precluding generation of an infectious virus. The disadvantage of nonviral delivery systems had been the low levels of delivery and gene expression produced by ''first-generation'' complexes. However, recent advances have dramatically improved transfection efficiencies and efficacy of liposomal vectors (14–18). Reviews of other in vivo delivery systems and improvements using cationic liposomes have been published recently (19,20).

Cationic liposome–nucleic acid complexes can be administered via numerous delivery routes in vivo. Routes of delivery include direct injection (e.g., intratumoral), intravenous, intraperitoneal, intra-arterial, intrasplenic, mucosal (nasal, vaginal, rectal), intramuscular, subcutaneous, transdermal, intradermal, subretinal, intratracheal, intracranial, and others. Much interest has focused on noninvasive intravenous administration because many investigators believe that this route of delivery is the ''holy grail'' for the treatment or cure of cancer, cardiovascular, and other inherited or acquired diseases. Particularly for the treatment of metastatic cancer, therapeutics must reach not only the primary tumor but also the distant metastases.

Optimization of cationic liposomal complexes for in vivo applications and therapeutics is complex, involving many distinct components. These components include nucleic acid purification, plasmid design, formulation of the delivery vehicle, administration route and schedule, dosing, detection of gene expression, and others. Often I make the analogy of liposome optimization to a functional car. Of course, the engine of the car, analogous to the liposome delivery vehicle, is extremely important. However, if the car does not have wheels, adequate tires, etc., the motorist will not be able to drive the vehicle to its destination. This chapter focuses on optimization of these distinct components for use in a variety of in vivo applications. Optimizing all components of the delivery system will allow broad use of liposomal complexes to treat or cure human diseases or disorders.

II. OPTIMIZATION OF CATIONIC LIPOSOME FORMULATIONS FOR USE IN VIVO

Much research has been directed toward the synthesis of new cationic lipids. Some new formulations led to the discovery of more efficient transfection agents for cells in culture. However, their efficiency measured in vitro did not correlate with their ability to deliver DNA after administration in animals. Functional properties defined in vitro do not assess the stability of the complexes in plasma or their pharmacokinetics and biodistribution, all of which are essential for optimal activity in vivo. Colloidal properties of the complexes in addition to the physicochemical properties of their component lipids also determine these parameters. In particular, in addition to efficient transfection of target cells, nucleic acid–liposome complexes must be able to traverse tight barriers in vivo and penetrate throughout the target tissue to produce efficacy for the treatment of disease. These are not issues for achieving efficient transfection of cells in culture with the exception of polarized tissue culture cells. Therefore, we are not surprised that optimized liposomal delivery vehicles for use in vivo may be different than those used for efficient delivery to cells in culture.

In summary, in vivo nucleic acid–liposome complexes that produce efficacy in animal models of disease have extended half-life in the circulation, are stable in serum, have broad biodistribution, efficiently encapsulate various sizes of nucleic acids, are targetable to specific organs and cell types, penetrate across tight barriers in several organs, penetrate evenly throughout the target tissue, are optimized for nucleic acid : lipid ratio and colloidal suspension in vivo, can be size fractionated to produce a totally homogenous population of complexes prior to injection, and can be repeatedly administered. Recently, we demonstrated efficacy of a robust liposomal delivery system in small and large animal models for lung (15), breast (17), head and neck (Hung and Templeton, 2002), and pancreatic cancers (16), and for hepatitis B and C (Clawson and Templeton, 2000). Based on efficacy in these animal studies, this liposomal delivery system will be used in upcoming clinical trials to treat these cancers. Our studies demonstrated broad efficacy in the use of liposomes to treat disease and have dispelled several myths that exist concerning the use of liposomal systems.

III. LIPOSOME MORPHOLOGY AND EFFECTS ON GENE DELIVERY AND EXPRESSION

Efficient in vivo nucleic acid–liposome complexes have unique features, including their morphology, mechanisms for crossing the cell membrane and entry into the nucleus, ability to be targeted for delivery to specific cell surface receptors, and ability to penetrate across tight barriers and throughout target tissues. Liposomes have different morphologies based on their composition and the formulation method. Furthermore, the morphology of complexes can contribute to their ability to deliver nucleic acids in vivo. Formulations frequently used for the delivery of nucleic acids are lamellar structures, including small unilamellar vesicles (SUVs), multilamellar vesicles (MLVs), or bilamellar invaginated vesicles (BIVs), recently developed in our laboratory (Fig. 1). Several investigators developed liposomal delivery systems using hexagonal structures; however,

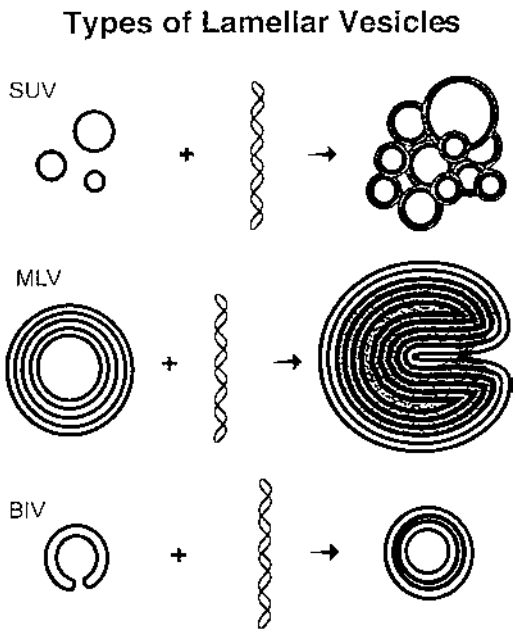

Figure 1 Diagrams drawn from cryoelectron micrographs of cross-sections through vitrified films of various types of liposomes and DNA–liposome complexes. SUVs condense nucleic acids on the surface and produce ''spaghetti and meatballs'' structures. MLVs appear as ''Swiss rolls'' after mixing with DNA. BIVs are produced using a formulation developed in our laboratory (14). Nucleic acids are efficiently encapsulated between 2 BIVs.

they demonstrated efficiency primarily for the transfection of some cell types in culture and not for in vivo delivery. SUVs condense nucleic acids on the surface and form ''spaghetti and meatballs'' structures (21). DNA–liposome complexes made using SUVs produce little or no gene expression upon systemic delivery, although these complexes transfect numerous cell types efficiently in vitro (1,2). Furthermore, SUV liposome–DNA complexes cannot be targeted efficiently. SUV liposome–DNA complexes also have a short half-life within the circulation, generally about 5 to 10 min. Polyethylene glycol (PEG) has been added to liposome formulations to extend their half-life (22–24); however, PEGylation created other problems that have not been resolved. PEG seems to hinder delivery of cationic liposomes into cells due to its sterically hindering ionic interactions, and it interferes with optimal condensation of nucleic acids onto the cationic delivery vehicle. Furthermore, extremely long half-life in the circulation (e.g., several days) has caused problems for patients because the bulk of the PEGylated liposomal formulation doxil that encapsulates the cytotoxic agent, doxorubicin, accumulates in the skin, hands, and feet. For example, patients contract mucositis and hand and foot syndrome (25,26) that cause extreme discomfort to the patient. Attempts to add ligands to doxil for delivery to specific cell surface receptors has not resulted in much cell-specific delivery, and the majority of the injected targeted formulation still accumulates in the skin, hands, and feet. Addition of PEG into formulations developed in our laboratory also caused steric hindrance in the bilamellar invaginated structures that did not encapsulate DNA efficiently, and gene expression was substantially diminished.

Some investigators have loaded nucleic acids within SUVs using a variety of methods; however, the bulk of the DNA does not load or stay within the liposomes. Furthermore, most of the processes used for loading nucleic acids within liposomes are extremely time consuming and not cost effective. Therefore, SUVs are not the ideal liposomes for creating nonviral vehicles for targeted delivery.

Complexes made using MLVs appear as ''Swiss rolls'' when viewing cross-sections by cryoelectron microscopy (27). These complexes can become too large for systemic administration or deliver nucleic acids inefficiently into cells due to inability to ''unravel'' at the cell surface. Addition of ligands onto MLV liposome–DNA complexes further aggravates these problems. Therefore, MLVs are not useful for the development of targeted delivery of nucleic acids.

Using a formulation developed in our laboratory, nucleic acids are efficiently encapsulated between 2 BIVs (14). We created these unique structures using 1,2-bis(oleoyloxy)-3-(trimethylammino)propane (DOTAP) and cholesterol (Chol), and a novel formulation procedure. This procedure is different because it includes a brief, low-frequency sonication, followed by manual extrusion through filters of decreasing pore size. The 0.1- and 0.2-um filters used are made of aluminum oxide and not polycarbonate, which is typically used by other protocols. Aluminum oxide membranes contain more pores per surface area, evenly spaced and sized pores, and pores with straight channels. During the manual extrusion process, the liposomes are passed through each of 4 different-size filters only once. This process produces 88% invaginated liposomes. Use of high-frequency sonication and/or mechanical extrusion produces only SUVs.

The BIVs produced condense unusually large amounts of nucleic acids of any size (Fig. 2) or viruses (Fig. 3). Furthermore, addition of other DNA-condensing agents including polymers is not necessary. For example, condensation of plasmid DNA onto polymers first before encapsulation in the BIVs did not increase condensation or subsequent gene expression after transfection in vitro or in vivo. Encapsulation of nucleic acids by these BIVs alone is spontaneous and immediate, and therefore, cost effective, requiring only 1 step of simple mixing. The extruded DOTAP : Chol–nucleic acid complexes are also large enough that they are not cleared rapidly by Kupffer cells in the liver, and yet extravasate across tight barriers, including the endothelial cell barrier of the lungs in a normal mouse, and diffuse through target organs efficiently (15). Our recent work demonstrating efficacy for treatment of nonsmall cell lung cancer (15) showed that only BIV DOTAP : Chol–p53 DNA liposome complexes produced efficacy, and SUV DOTAP:Chol–p53 DNA liposome complexes produced no efficacy. Therefore, the choice of lipids alone is

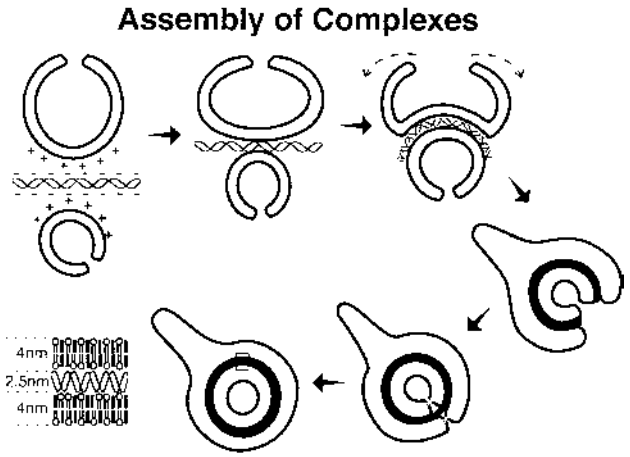

Figure 2 Proposed model showing cross-sections of extruded DOTAP:Chol liposomes (BIVs) interacting with nucleic acids. Nucleic acids adsorb onto a BIV via electrostatic interactions. Attraction of a second BIV to this complex results in further charge neutralization. Expanding electrostatic interactions with nucleic acids cause inversion of the larger BIV and total encapsulation of the nucleic acids. Inversion can occur in these liposomes because of their excess surface area, which allows them to accommodate the stress created by the nucleic acid–lipid interactions. Nucleic acid binding reduces the surface area of the outer leaflet of the bilayer and induces the negative curvature due to lipid ordering and reduction of charge repulsion between cationic lipid headgroups. Condensation of the internalized nucleic acid-lipid sandwich expands the space between the bilayers and may induce membrane fusion to generate the apparently closed structures. The enlarged area shows the arrangement of nucleic acids condensed between two 4-nm bilayers of extruded DOTAP:Chol.

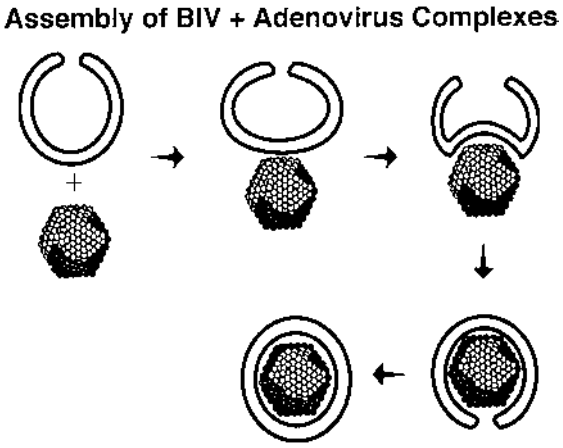

Figure 3 Proposed model showing cross-sections of an extruded DOTAP:Chol liposome (BIV) interacting with adenovirus. Adenovirus interacts with a BIV, causing negative curvature and wrapping around the virus particle.

not sufficient for optimal DNA delivery, and the morphology of the complexes is essential.

IV. OPTIMAL LIPIDS AND LIPOSOME MORPHOLOGY: EFFECTS ON GENE DELIVERY AND EXPRESSION

Choosing the best cationic lipids and neutral lipids are also essential for producing the optimal in vivo formulation. For example, using our novel manual extrusion procedure does not produce BIVs using the cationic lipid dimethyldioctadecylammonium bromide (DDAB). Furthermore, DOTAP is biodegradable, whereas DDAB is not biodegradable. Use of biodegradable lipids is preferred for use in humans. Furthermore, only DOTAP and not DDAB-containing liposomes produced highly efficient gene expression in vivo (14). DDAB did not produce BIVs and was unable to encapsulate nucleic acids. Apparently, DDAB- and DOTAP-containing SUVs produce similar efficiency of gene delivery in vivo; however, these SUVs are not as efficient as BIV DOTAP:Chol (14). In addition, use of L-α-dioleoyl phosphatidylethanolamine (DOPE) as a neutral lipid creates liposomes that cannot wrap or encapsulate nucleic acids. Several investigators have reported efficient transfection of cells in culture using DOPE in liposomal formulations. However, our data showed that formulations consisting of DOPE were not efficient for producing gene expression in vivo (14).

Investigators must also consider the source and lot of certain lipids purchased from companies. For example, different lots of cholesterol from the same vendor can vary dramatically and will affect the formulation of liposomes. Recently, we are using synthetic cholesterol (Sigma, St. Louis, MO). Synthetic cholesterol, instead of natural cholesterol purified from the wool of sheep, is preferred by the Food and Drug Administration for use in producing therapeutics for injection into humans.

Our BIV formulations are also stable for a few years as liquid suspensions. Freeze-dried formulations that are stable indefinitely even at room temperature can also be made. Stability of liposomes and liposomal complexes is also essential, particularly for the commercial development of human therapeutics.

V. LIPOSOME ENCAPSULATION, FLEXIBILITY, AND OPTIMAL COLLOIDAL SUSPENSIONS

A common belief is that artificial vehicles must be 100 nm or smaller to be effective for systemic delivery. However, this belief is most likely true only for large, inflexible delivery vehicles. Blood cells are several microns (up to 7000 nm) in size and yet have no difficulty circulating in the blood, includ-

ing through the smallest capillaries. However, sickle-cell blood cells, which are rigid, do have problems in the circulation. Therefore, we believe that flexibility is a more important issue than small size. In fact, BIV DNA–liposome complexes in the size range of 200 to 450 nm produced the highest levels of gene expression in all tissues after intravenous injection (14). Delivery vehicles, including nonviral vectors and viruses, which are not PEGylated and are smaller than 200 nm, are cleared quickly by the Kupffer cells in the liver. Therefore, increased size of liposomal complexes could extend their circulation time, particularly when combined with injection of high colloidal suspensions. BIVs are able to encapsulate nucleic acids and viruses, apparently due to the presence of cholesterol in the bilayer (Fig. 4). Whereas formulations including DOPE instead of cholesterol could not assemble nucleic acids by a ''wrapping type'' of mechanism (Fig. 5), and produced little gene expression in the lungs and no expression in other tissues after intravenous injections. Because the extruded DOTAP:Chol BIV complexes are flexible and not rigid, are stable in high concentrations of serum, and have extended half-life, they do not have difficulty circulating efficiently in the bloodstream.

We believe that colloidal properties of nucleic acid–liposome complexes also determine the levels of gene expression produced after in vivo delivery (14,28). These properties include the DNA:lipid ratio that determines the overall charge density of the complexes and the colloidal suspension that is monitored by its turbidity. Complex size and shape, lipid composition and formulation, and encapsulation efficiency of nucleic acids by the liposomes also contribute to the colloidal properties of the complexes. The colloidal properties affect serum stability, protection from nuclease degradation, blood circulation time, and biodistribution of the complexes.

Our in vivo transfection data showed that an adequate amount of colloids in suspension was required to produce efficient gene expression in all tissues examined (14). The colloidal suspension is assessed by measurement of adsorbance at 400 nm using a spectrophotometer optimized to measure turbidity. Our data showed that transfection efficiency in all tissues corresponded to OD400 of the complexes measured prior to intravenous injection.

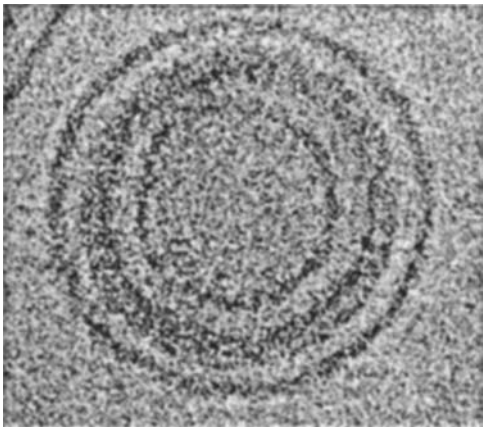

Figure 4 Cryoelectron micrograph of BIV DOTAP:Chol–DNA liposome complexes. The plasmid DNA is encapsulated between 2 BIVs.

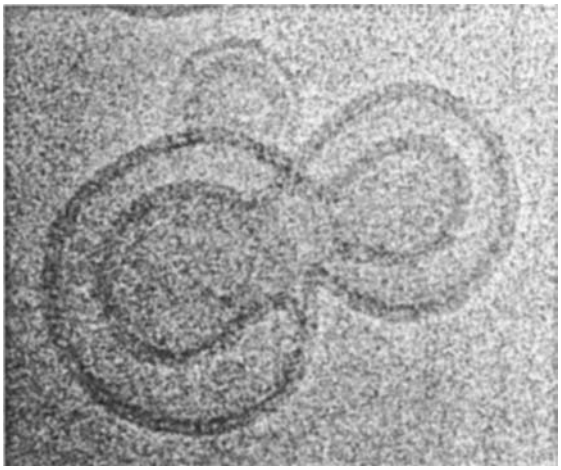

Figure 5 Cryoelectron micrograph of extruded DOTAP:DOPE liposomes complexed to plasmid DNA. Although these liposomes were prepared by the same protocol that produces BIV DOTAP:Chol, these vesicles cannot wrap and encapsulate nucleic acids. The DNA condenses on the surfaces of the liposomes shown.

VI. OVERALL CHARGE OF COMPLEXES AND ENTRY INTO THE CELL

In addition, our delivery system is efficient because the complexes deliver DNA into cells by fusion with the cell membrane and avoid the endocytic pathway (Fig. 6). Cells are negatively charged on the surface, and specific cell types vary in their density of negative charge. These differences in charge density can influence the ability of cells to be transfected. Cationic complexes have nonspecific ionic charge interactions with cell surfaces. Efficient transfection of cells by cationic complexes is, in part, contributed by adequate charge interactions. In addition, recent publications report that certain viruses have a partial positive charge around key subunits of viral proteins on the virus surface responsible for binding to and internalization through target cell surface receptors. Therefore, this partial positive charge is required for virus entry into the cell. Thus, maintenance of adequate positive charge on the surface of targeted liposome complexes is essential for optimal delivery into the cell. Different formulations of liposomes interact with cell surfaces via a variety of mechanisms. Two major pathways for interaction are by endocytosis or by direct fusion with the cell membrane (27,29–34). Preliminary data suggest that nucleic acids delivered in vitro and

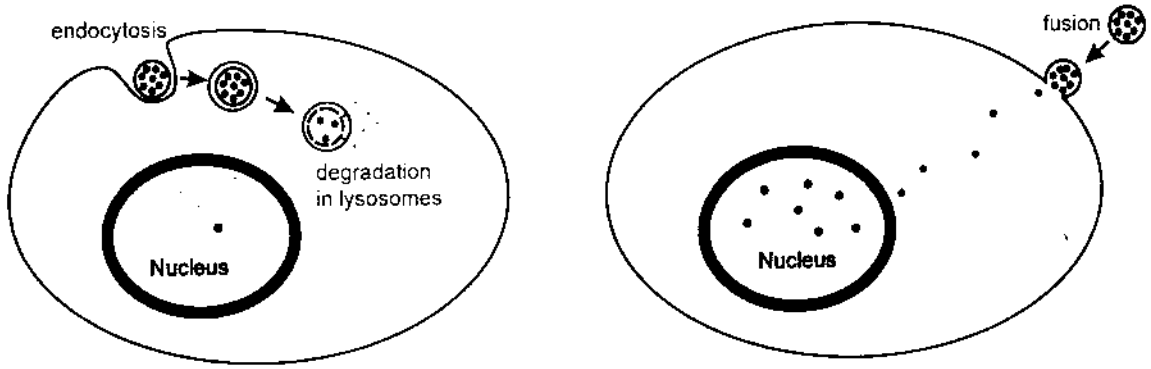

Figure 6 Mechanisms for cell entry of nucleic acid–liposome complexes. Two major pathways for interaction are by endocytosis or by direct fusion with the cell membrane. Complexes that enter the cell by direct fusion allow delivery of more nucleic acids to the nucleus because the bulk of the nucleic acids do not enter endosomes.

in vivo using complexes developed in our lab enter the cell by direct fusion (Fig. 6). Apparently, the bulk of the nucleic acids do not enter endosomes, and therefore, far more nucleic acid enters the nucleus. Cell transfection by direct fusion produced orders of magnitude, increased levels of gene expression, and numbers of cells transfected vs. cells transfected through the endocytic pathway.

We believe that maintenance of adequate positive charge on the surface of complexes is essential to drive cell entry by direct fusion. Therefore, we create targeted delivery of our complexes in vivo without the use of PEG. These ligand-coated complexes also reexpose the overall positive charge of the complexes as they approach the target cells. Through ionic interactions or covalent attachments, we have added monoclonal antibodies, Fab fragments, proteins, partial proteins, peptides, peptide mimetics, small molecules, and drugs to the surface of our complexes after mixing. These ligands efficiently bind to the target cell surface receptor, and maintain entry into the cell by direct fusion. Using novel methods for addition of ligands to the complexes for targeted delivery results in further increased gene expression in the target cells after transfection. Therefore, we design targeted liposomal delivery systems that retain predominant entry into cells by direct fusion vs. the endocytic pathway. Figure 7 shows our optimized strategy to achieve targeted delivery, deshielding, fusion with the cell membrane, entry of nucleic acids into the cell and to the nucleus, and production of gene expression of a cDNA cloned in a plasmid.

VII. SERUM STABILITY OF OPTIMIZED NUCLEIC ACID–LIPOSOME COMPLEXES FOR USE IN VIVO

Serum stability of cationic complexes is complicated and cannot be assessed by simply performing studies at a random concentration of serum. Figure 8 shows results from serum stability studies of DNA–liposome complexes that have been optimized in our laboratory for systemic delivery. Serum stability of these complexes was studied at 37°C out to 24 h at concentrations of serum ranging from 0% to 100%. Two different serum stability assays were performed. The first assay measured the OD400 of BIV DOTAP:Chol–DNA liposome complexes added into tubes containing a different concentration of serum in each tube, ranging from 0% to 100%. The tubes were incubated at 37°C, and small aliquots from each tube were removed at various time points out to 24 h. The OD400 of each aliquot was measured on a spectrophotometer calibrated to accurately measure turbidity. Previous work in our laboratory demonstrated that the OD400 predicted both the stability of the complexes and the transfection efficiency results obtained for multiple organs after intravenous injections (14,28). Percent stability for this assay is defined as the transfection efficiency that is obtained at a particular OD400 of the complexes used for intravenous injections. Therefore, this assay is rigorous because slight declines in OD400 of these complexes result in obtaining no transfection in vivo. Declines in the OD400 also measure precipitation of the complexes.

A second assay was performed to support the results obtained from the OD400 measurements described above. A different concentration of serum, ranging from 0% to 100%, was placed into each well of a 96-well microtiter dish. BIV DOTAP:Chol–DNA liposome complexes were added to the serum in the wells, and the plate was incubated at 37°C. The plate was removed at various time points out to 24 h and complexes in the wells were observed under the microscope. Precipitation of complexes in the wells was assessed. Stability (100%) was set at no precipitation observed. Results from this assay were compared with those obtained in the first assay. Stability (100%) of complexes was set at no decline of OD400 in assay #1 and no observed precipitation in assay #2 at each % serum concentration, and the results were plotted (Fig. 8).

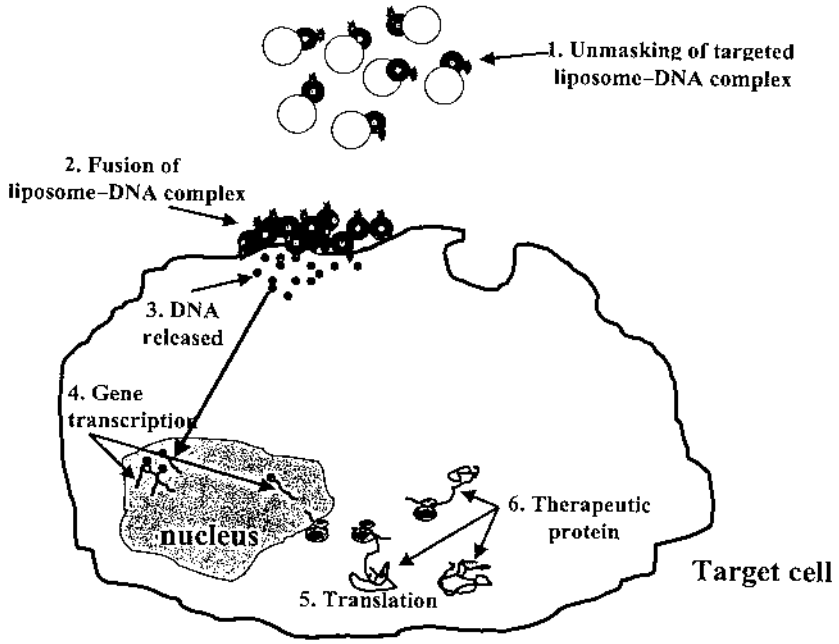

Figure 7 Optimized strategy for delivery and gene expression in the target cell. Optimization of many steps is required to achieve targeted delivery, deshielding, fusion with the cell membrane, entry of nucleic acids into the cell and to the nucleus, and production of gene expression of a cDNA cloned in a plasmid.

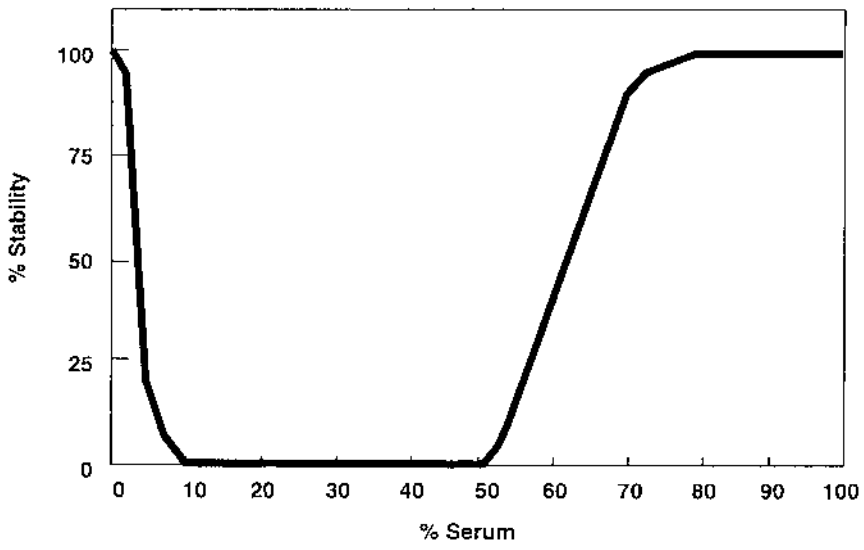

Figure 8 Serum stability profile for DNA–liposome complexes optimized for systemic delivery. Serum stability of these complexes was studied at 37°C out to 24 h at concentrations of serum ranging from 0% to 100%. Serum stability at the highest concentrations of serum, about 70% to 100%, that are physiological concentrations of serum found in the bloodstream is required.

The results showed serum stability at the highest concentrations of serum, about 70% to 100%, that are physiological concentrations of serum found in the bloodstream. In addition, these complexes were also stable in no or low concentrations of serum, whereas the complexes were unstable at 10% to 50% serum, perhaps due to salt bridging. Therefore, in vitro optimization of serum stability for formulations of cationic complexes must be performed over a broad range of serum concentration to be useful for applications in vivo.

VIII. OPTIMIZED HALF-LIFE IN THE CIRCULATION

As stated above, the extruded BIV DOTAP:Chol–nucleic acid complexes are large enough so that they are not cleared rapidly by Kupffer cells in the liver and yet extravasate across tight barriers and diffuse through the target organ efficiently. Further addition of ligands to the surface of extruded BIV DOTAP:Chol–nucleic acid complexes does not significantly increase the mean particle size. Extravasation and penetration through the target organ and gene expression produced after transfection are not diminished. These modified formulations are positively charged and deliver nucleic acids efficiently into cells in vitro and in vivo. Because extruded BIV DOTAP:Chol–nucleic acid complexes with or without ligands have a 5-h half-life in the circulation, these complexes do not accumulate in the skin, hands, or feet. Extended half-life in the circulation is provided primarily by the formulation, preparation method, injection of optimal colloidal suspensions, and optimal nucleic acid:lipid ratio used for mixing complexes, serum stability, and size (200–450 nm). Therefore, these BIVs are ideal for use in the development of effective, targeted nonviral delivery systems that clearly require encapsulation of nucleic acids.

IX. BROAD BIODISTRIBUTION OF OPTIMIZED LIPOSOME FORMULATIONS

Our ''generic'' BIV nucleic acid–liposome formulation transfects many organs and tissues efficiently after intravenous injection (14) and has demonstrated efficacy in animal models for lung cancer (15), pancreatic cancer (17), breast cancer (16), hepatitis B and C (Clawson and Templeton, 2000), and cardiovascular diseases (35). Therefore, optimization of the morphology of the complexes, the lipids used, flexibility of the liposomes and complexes, colloidal suspension, overall charge, serum stability, and half-life in circulation allows for efficient delivery and gene expression in many organs and tissues other than the lung. Apparently, these extruded DOTAP:Chol BIV nucleic acid–liposome complexes can overcome the tendency to be adsorbed only by the endothelial cells lining the circulation surrounding the lungs described by other investigators (36). However, as discussed above and below, we can further direct delivery to specific target tissues or cells by our targeted delivery strategies in combination with reversible masking used to bypass nonspecific transfection.

X. OPTIMIZATION OF TARGETED DELIVERY

Much effort has been made to specifically deliver nucleic acid–liposome complexes to target organs, tissues, and/or cells. Ligands that bind to cell surface receptors are usually attached to PEG and then attached to the cationic or anionic delivery vehicle. Due to shielding the positive charge of cationic complexes by PEG, delivery to the specific cell surface receptor can be accomplished by only a small fraction of complexes injected systemically. Furthermore, delivery of PEGylated complexes into the cell occurs predominantly through the endocytic pathway, and subsequent degradation of the bulk of the nucleic acid occurs in the lysosomes. Thus, gene expression is generally lower in the target cell than using the nonspecific delivery of highly efficient cationic complexes.

As discussed above, the vast majority of the injected PEGylated complexes bypasses the target cell. Apparently, the PEGylated complexes cannot use critical charge interactions for optimal transfection into cells by direct fusion. Inability to expose positive charge on the surface of optimized complexes results in the transfection of fewer cells. PEGylation was first used to increase the half-life of complexes in the circulation and to avoid uptake in the lung. However, this technology also destroys the ability to efficiently transfect cells. We were able to increase the half-life in circulation of BIVs to 5 h without the use of PEG. Because the extended half-life of BIVs is not too long, this delivery system does not result in the accumulation of complexes in nontarget tissues that circulate for 1 to 3 days. Some investigators have now reported targeted delivery that produces increased gene expression in the target cell over their nontargeted complexes. However, these nontargeted and targeted delivery systems are inefficient (37) compared with efficient delivery systems such as the BIVs.

In using the extruded BIV DOTAP:Chol nucleic acid:liposome complexes, we produced an optimal half-life in the circulation without the use of PEG (14). Extended half-life was produced primarily by the formulation, preparation method, injection of optimal colloidal suspensions, serum stability, and optimal nucleic acid:lipid ratio used for mixing complexes, and size (200–450 nm). Furthermore, we avoid uptake in the lungs using the negative charge of the ligands and ''shielding/deshielding compounds'' that can be added to the complexes used for targeting just prior to injection or administration in vivo. Our strategy to bypass nonspecific transfection is called reversible masking. By adding ligands using the novel approaches that we developed, adequate overall positive charge on the surface of complexes is preserved. In summary, we achieve optimal circulation time of the complexes, reach and deliver to the target organ, avoid uptake in nontarget tissues, and efficiently interact with the cell surface to produce optimal transfection.

XI. EFFICIENT DISSEMINATION THROUGHOUT TARGET TISSUES AND MIGRATION ACROSS TIGHT BARRIERS

A primary goal for efficient in vivo delivery is to achieve extravasation into and penetration throughout the target organ/tissue ideally by noninvasive systemic administration. Without these events therapeutic efficacy is highly compromised for any treatment including gene and drug therapies. Achieving this goal is difficult due to the many tight barriers that exist in animals and people. Furthermore, many of these barriers become tighter in the transition from neonates to becoming adults. Penetration throughout an entire tumor is further hindered due to the increased interstitial pressure within most tumors (38–40). We believe that nonviral systems can play a pivotal role in achieving target organ extravasation and penetration needed to treat or cure certain diseases. Our preliminary studies have shown that extruded BIV DOTAP:Chol nucleic acid:liposome complexes can extravasate across tight barriers and penetrate evenly throughout entire target organs, and viral vectors cannot cross identical barriers. These barriers include the endothelial cell barrier in a normal mouse, the posterior blood retinal barrier in adult mouse eyes, complete and even diffusion throughout large tumors (15), and penetration through several tight layers of smooth muscle cells in the arteries of pigs (35). Diffusion throughout large tumors was measured by expression of β-galactosidase or the proapoptotic gene p53 in about half of the p53-null tumor cells after a single injection of BIV DOTAP:Chol–DNA liposome complexes into the center of a tumor. Transfected cells were evenly spread throughout the tumors. Tumors injected with complexes encapsulating plasmid DNA encoding p53 showed apoptosis in almost all the tumor cells by TUNEL staining. Tumor cells expressing p53 mediate a bystander effect on neighboring cells perhaps due to up-regulation by Fas ligand that causes nontransfected tumor cells to undergo apoptosis. Currently, we are investigating the mechanisms used by extruded DOTAP:Chol nucleic acid:liposome complexes to cross-barriers and penetrate throughout target organs. By knowing more about these mechanisms, we hope to develop more robust nonviral gene therapeutics.

XII. OPTIMIZATION OF PLASMIDS FOR IN VIVO GENE EXPRESSION

Delivery of DNA and subsequent gene expression may be poorly correlated (18,41). Investigators may focus solely on the delivery formulation as the source of poor gene expression. In many cases, however, the delivery of DNA into the nucleus of a particular cell type may be efficient, although little or no gene expression is achieved. The causes of poor gene expression can be numerous. The following issues should be considered independent of the delivery formulation, including suboptimal promoter enhancers in the plasmid, poor preparation of plasmid DNA, and insensitive detection of gene expression.

Plasmid expression cassettes typically have not been optimized for animal studies. For example, many plasmids lack a full-length cytomegalovirus (CMV) promoter enhancer. Over 100 variations of the CMV promoter enhancer exist, and some variations produce greatly reduced or no gene expression in certain cell types (18). Even commercially available plasmids contain suboptimal CMV promoters enhancers, although these plasmids are advertised for use in animals. Furthermore, upon checking the company data for these plasmids, one would discover that these plasmids have never been tested in animals and have been tested in only 1 or 2 cultured cell lines. Conversely, plasmids that have been optimized for overall efficiency in animals may not be best for transfection of certain cell types in vitro or in vivo. For example, many investigators have shown that optimal CMV promoters enhancers produce gene expression at levels several orders of magnitude less in certain cell types. In addition, one cannot assume that a CMV promoter that expresses well within the context of a viral vector, such as adenovirus, will function as well in a plasmid-based transfection system for the same cell context. Virus proteins produced by the viral vector are required for producing high levels of mRNA by the CMV promoter in specific cell nuclei.

Ideally, investigators design custom promoter enhancer chimeras that produce the highest levels of gene expression in their target cells of interest. Recently, we designed a systematic approach for customizing plasmids used for breast cancer gene therapy using expression profiling (18). Gene therapy clinical trials for cancer frequently produce inconsistent results. We believe that some of this variability could result from differences in transcriptional regulation that limit expression of therapeutic genes in specific cancers. Our systemic liposomal delivery of a nonviral plasmid DNA showed efficacy in animal models for several cancers. However, we observed large differences in the levels of gene expression from a CMV promoter enhancer between lung and breast cancers. To optimize gene expression in breast cancer cells in vitro and in vivo, we created a new promoter enhancer chimera to regulate gene expression. Serial analyses of gene expression data from a panel of breast carcinomas and normal breast cells predicted promoters that are highly active in breast cancers [e.g., the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter]. Furthermore, GAPDH is up-regulated by hypoxia, which is common in tumors. We added the GAPDH promoter, including the hypoxia enhancer sequences, to our in vivo gene expression plasmid. The novel CMV-GAPDH promoter enhancer showed up to 70-fold increased gene expression in breast tumors compared with the optimized CMV promoter enhancer alone. No significant increase in gene expression was observed in other tissues. These data demonstrate tissue-specific effects on gene expression after nonviral delivery and suggest that gene delivery systems may require plasmid modifications for the treatment of different tumor types. Furthermore, expression profiling can facilitate the design of optimal expression plasmids for use in specific cancers.

Several reviews have stated that nonviral systems are intrinsically inefficient compared with viral systems. However, as discussed above, one must separate issues of the delivery

vehicle vs. the plasmid that is delivered. Case in point, we have shown that our extruded liposomes optimized for systemic delivery could out-compete delivery using a lentivirus. For example, we have compared SIVmac239, a highly noninfectious virus, with nonviral delivery of SIVmac239 DNA complexed to BIVs in adult rhesus macaques after injection into the saphenous vein of the leg. Our data showed that the monkeys injected with SIV DNA encapsulated in DOTAP:Chol BIVs were infected 4 days postinjection, and high levels of infection were produced in these monkeys at 14 days postinjection. Furthermore, higher levels of SIV RNA in the blood were produced using our BIV liposomes for delivery vs. using the SIV virus. CD4 counts were measured before and after injections. CD4 levels dropped in all monkeys to the lowest levels ever detected in the macaques in any experiment by 28 days postinjection, the first time point at which these counts were measured postinjection. All monkeys had clinical SIV infections and lost significant weight by day 28. These results were surprising because SIVmac239 is not highly infectious, and monkeys become sick with SIV infection only after several months or years postinjection with SIVmac239 virus. Therefore, we were able to induce SIV infection faster using our nonviral delivery of SIV plasmid DNA. In this case, we delivered a replication-competent plasmid so that gene expression increased over time posttransfection. Our delivery system was highly efficient and exceeded that of the lentivirus. The critical feature in this nonviral experiment was the plasmid DNA that was delivered.

Plasmids can be engineered to provide for specific or long-term gene expression, replication, or integration. Persistence elements, such as the inverted terminal repeats from adenovirus or adeno-associated virus, have been added to plasmids to prolong gene expression in vitro and in vivo. Apparently, these elements bind to the nuclear matrix, thereby retaining the plasmid in cell nuclei. For regulated gene expression, many different inducible promoters are used that promote expression only in the presence of a positive regulator or in the absence of a negative regulator. Tissue-specific promoters have been used for the production of gene expression exclusively in the target cells. As discussed in the previous paragraph, replication-competent plasmids or plasmids containing sequences for autonomous replication can be included that provide prolonged gene expression. Other plasmid-based strategies produce site-specific integration or homologous recombination within the host cell genome [reviewed in (42)]. Integration of a cDNA into a specific ''silent site'' in the genome could provide long-term gene expression without disruption of normal cellular functions. Homologous recombination could correct genetic mutations upon integration of wild-type sequences that replace mutations in the genome. Plasmids that contain fewer bacterial sequences and that produce high yield upon growth in *Escherichia coli* are also desirable.

XIII. OPTIMIZATION OF PLASMID DNA PREPARATIONS

The transfection quality of plasmid DNA is dependent on the preparation protocol and training of the person preparing the DNA. For example, we performed a blinded study asking 3 people to make DNA preparations of the same plasmid from the same box of a Qiagen Endo-Free Plasmid Preparation kit. One person then mixed all the DNA–liposome complexes on the same morning using a single vial of liposomes. One person performed all tail vein injections, harvesting of tissues, preparation of extracts from tissues, and reporter gene assays on the tissue extracts. In vivo gene expression differed 30-fold among these 3 plasmid DNA preparations.

One source for this variability is that optimized methods to detect and remove contaminants from plasmid DNA preparations have not been available. We have identified large amounts of contaminants that exist in laboratory and clinical grade preparations of plasmid DNA. These contaminants copurify with DNA by anion exchange chromatography and by cesium chloride density gradient centrifugation. Endotoxin removal does not remove these contaminants. High-performance liquid chromatography cannot detect these contaminants. Therefore, we developed 3 proprietary methods for the detection of these contaminants in plasmid DNA preparations. We can now make clinical grade (GMP) DNA that does not contain these contaminants. To provide the greatest efficacy and levels of safety, these contaminants must be assessed and removed from plasmid DNA preparations. These contaminants belong to a class of molecules known to inhibit both DNA and RNA polymerase activities. Therefore, gene expression posttransfection can be increased by orders of magnitude if these contaminants are removed from DNA preparations. The presence of these contaminants in DNA also precludes high-dose delivery of DNA–liposome complexes intravenously. Our group and other investigators have shown that intravenous injections of high doses of improved liposomes alone cause no adverse effects in small and large animals.

Some investigators have removed the majority of CpG sequences from their plasmids and report reduced toxicity after intravenous injections of cationic liposomes complexed to these plasmids (43). However, only low doses containing up to 16.5 μg of DNA per injection into each mouse were shown to reduce toxicity. To achieve efficacy for cancer metastases, particularly in mice bearing aggressive tumors, most investigators are interested in injecting higher doses in the range of 50 to 150 μg of DNA per mouse. Therefore, removal of CpG sequences from plasmid-based gene therapy vectors will not be useful for these applications because no difference in toxicity was shown after intravenous injections of these higher doses of plasmids, with or without reduced CpG sequences, complexed to liposomes (43). Therefore, we believe that removal of the other contaminants in current DNA preparations, discussed above, is the major block to the safe intravenous injection of high doses of DNA–liposome complexes.

XIV. DETECTION OF GENE EXPRESSION

Thought should also be given to choosing the most sensitive detection method for every application of nonviral delivery rather than using the method that seems most simple. For

example, detection of β-galactosidase expression is far more sensitive than that for the green fluorescent protein (GFP). Specifically, 500 molecules of β-galactosidase (β-gal) per cell are required for detection using X-gal staining, whereas about 1 million molecules of GFP per cell are required for direct detection. Furthermore, detection of GFP may be impossible if the fluorescence background of the target cell or tissue is too high. Detection of chloramphenicol acetyltransferase (CAT) is extremely sensitive with little or no background detected in untransfected cells. Often, assays for CAT expression can provide more useful information than using β-gal or GFP as reporter genes.

Few molecules of luciferase in a cell can be detected by luminescence assays of cell or tissue extracts posttransfection. The sensitivity of these assays is highly dependent on the type of instrument used to measure luminescence. However, luciferase results may not predict the therapeutic potential of a nonviral delivery system. For example, if several hundred or thousand molecules per cell of a therapeutic gene are required to produce efficacy for a certain disease, then production of only few molecules will not be adequate. If only few molecules of luciferase are produced in the target cell using a specific nonviral delivery system, then the investigator may be misled in using this system for therapeutic applications.

Furthermore, noninvasive detection of luciferase expression in vivo is not as sensitive as luminescence assays of cell or tissue extracts posttransfection. Recently, my colleagues tried cooled charge-coupled device (CCD) camera imaging on live mice after intravenous injection of other cationic liposomes complexed to plasmid DNA-encoding luciferase, and they were not able to detect any transfection. However, these liposomal delivery systems had been used to detect luciferase by luminescence assays of organ extracts. My colleagues detected luciferase expression by CCD imaging after intravenous injections of BIV DOTAP:Chol–luciferase DNA–liposome complexes (44). Because the luciferase protein is short-lived, maximal expression was detected at 5 h posttransfection, whereas detection of herpes simplex virus-thymidine kinase (HSV-TK) gene expression using microPET imaging in the same mice was highest at 24 h posttransfection. In contrast to luciferase, the CAT protein accumulates over time, and therefore, the investigator is not restricted to a narrow time frame for assaying gene expression. Furthermore, detection of CAT seems to be more sensitive than CCD imaging of luciferase following intravenous injections of DNA–liposome complexes. However, the animals must be sacrificed in order to perform CAT assays on tissue or organ extracts. In summary, further work is still needed to develop in vivo detection systems that have high sensitivity and low background.

XV. OPTIMIZATION OF DOSE AND FREQUENCY OF ADMINISTRATION

To establish the maximal efficacy for the treatment of certain diseases or for the creation of robust vaccines, injections, or administrations of the nonviral gene therapeutic, etc., via different routes may be required. For particular treatments, one should not assume that one delivery route is superior to others without performing the appropriate animal experiments. In addition, people with the appropriate expertise should perform the injections and administrations. In our experience, only a minority of people who claim expertise in performing tail vein injections can actually perform optimal injections.

The optimal dose should be determined for each therapeutic gene or other nucleic acid that is administered. The investigator should not assume that the highest tolerable dose is optimal for producing maximal efficacy. The optimal administration schedule should also be determined for each therapeutic gene or other nucleic acid. To progress faster, some investigators have simply used the same administration schedule that they used for chemotherapeutics, for example. The investigator should perform in vivo experiments to determine when gene expression and/or efficacy drops significantly. Most likely, readministration of the nonviral gene therapeutic is not necessary until this drop occurs. Loss of the therapeutic gene product will vary with the half-life of the protein produced. Therefore, if a therapeutic protein has a longer half-life, then the gene therapy could be administered less frequently.

XVI. SUMMARY

Overcoming some hurdles remain in the broad application of nonviral delivery, however, we are confident that we will successfully accomplish the remaining challenges soon. Furthermore, we predict that eventually the majority of gene therapies will use artificial reagents that can be standardized and regulated as drugs rather than biologics. We will continue to incorporate the molecular mechanisms of viral delivery that produce efficient delivery to cells into artificial systems. Therefore, the artificial systems, including liposomal delivery vehicles, will be further engineered to mimic the most beneficial parts of the viral delivery systems while circumventing their limitations. We will also maintain the numerous benefits of the liposomal delivery systems discussed in this chapter.

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17

Molecular Interactions in Lipids, DNA, and DNA–Lipid Complexes

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I. INTRODUCTION

Designed by nature for information and valued by molecular biologists for manipulation, DNA is also a favorite of physical chemists and physicists (1). Its mechanical properties (2), its interactions with other molecules (3), and its modes of packing (4) present tractable but challenging problems whose answers have in vivo and in vitro consequences. In the context of DNA transfection and gene therapy (5), what has been learned about molecular mechanics, interaction, and packing might teach us how to package DNA for more effective gene transfer. Among these modes of in vitro packaging are association with proteins, treatment with natural or synthetic cationic ''condensing agents,'' and combination with synthetic positively charged lipids (6).

In vivo, DNA is tightly held, not at all like the dilute solution form often studied in vitro (Fig. 1). This tight assembly necessarily incurs huge energetic costs of confinement, costs that create a tension under which DNA is expected to ravel or unravel its message. Through direct measurement of forces between DNA molecules (7) and direct observation of its modes of packing (8), we might see not only how to use concomitant energies to design better DNA transfer systems, but also how to better understand the sequences of events by which DNA is read in cells.

What binds these structures? To first approximation, for large, flexible biological macromolecules, the relevant interactions resemble those found among colloidal particles (9), where the size of the molecule (e.g., DNA molecules, lipid membranes, actin bundles) distinguishes it from simpler, smaller species (e.g., small solutes or salt ions). On the colloidal scale of tens of nanometers [1 nm $= 10^{-9}$m], only the interactions between macromolecules are evaluated explicitly, whereas the small molecular species only ''dress'' the large molecules and drive the interactions between them.

The electrical charge patterns of multivalent ions such as Mn^{+2}, Co^{3+}, or spermine^{+4} cation binding to negative DNA create attractive electrostatic and/or solvation forces that move DNA double helices to finite separations, despite the steric knock of DNA thermal Brownian motion (10). Solvation patterns about the cation-dressed structures create solvation forces: DNA–DNA repulsion because of water clinging to the surface, and attraction from the release of solvent (11). Positively charged histones will spool DNA into carefully distributed skeins, themselves arrayed for systematic unraveling and reading (12). Viral capsids will encase DNA, stuffed against its own DNA–DNA electrostatic and solvation repulsion, to keep it under pressure for release upon infection (13). In artificial preparations, the glue of positively charged and

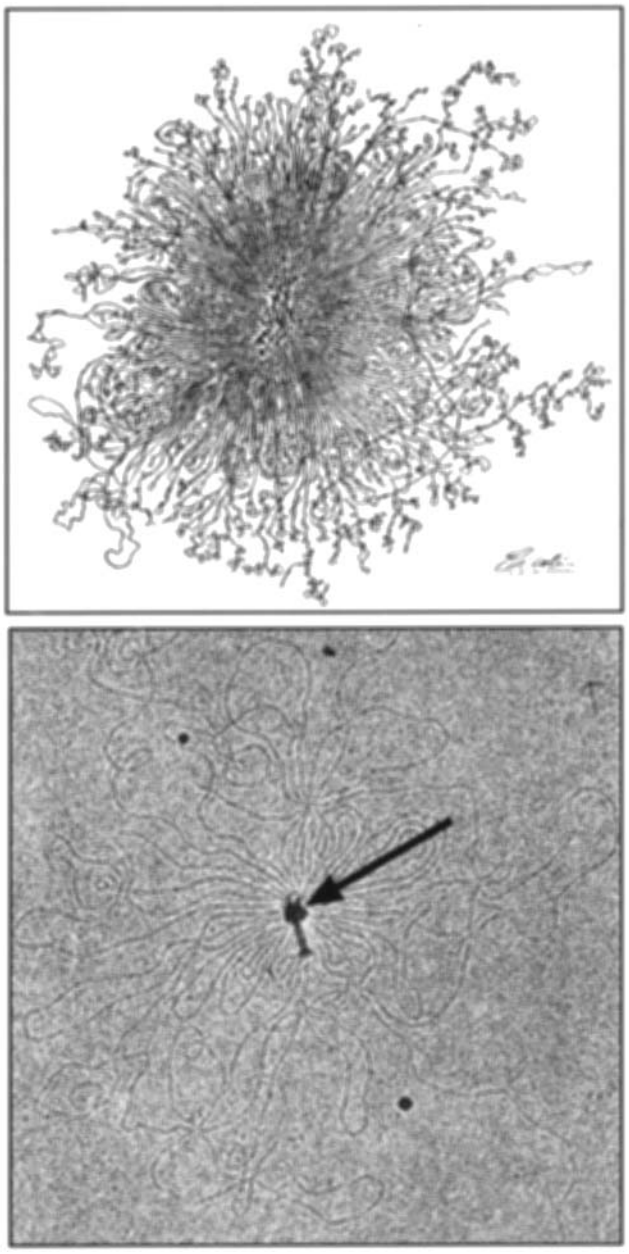


Figure 1 In vivo DNA is highly compacted. The figure shows *E. coli* DNA and T2 bacteriophage DNA after an osmotic shock in distilled water that has allowed them to expand from their much more compacted in vivo configurations. (*E. coli* picture courtesy of Ruth Kavenoff, Bluegenes, Inc., Los Angeles (1994); T2 picture from Kleinschmidt et al. BBA **61** (1962) 252.)

neutral lipids can lump negative DNA into ordered structures that can move through lipids and water solutions (14).

Changes in the suspending medium can modulate intermolecular forces. One example is the change in van der Waals charge fluctuation forces (see below) between lipid bilayers when small sugars modifying the dielectric dispersion properties of water are added to the solution (15). More dramatic, the addition of salt to water can substantially reduce electrostatic interactions between charged molecules such as DNA or other charged macromolecules bathed by an aqueous solution (16). These changes can modify the behavior of macromolecules quantitatively or induce qualitatively new features into their repertoire among these, most notably, precipitation of DNA by addition of organic polycations to the solution (10).

Similar observations can be made about the small molecules essential to practically every aspect of interaction between macromolecules. Through the dielectric constant or dielectric permittivity, it enters electrostatic interactions; through pH, it enters charging equilibria; and through its fundamental molecular geometry, it enters the hydrogen bond network topology around simple solutes. This is, of course, the water molecule (17). In what follows, we limit ourselves to only three basic properties of macromolecules—charge, polarity (solubility), and conformational flexibility—that appear to govern the plethora of forces encountered in biological milieu. It is no surprise that the highly ordered biological structures, such as the quasicrystalline spooling of DNA in viral heads or the multilamellar stacking of lipid membranes in visual receptor cells (Fig. 2), can be explained through the properties of a small number of fundamental forces acting between macromolecules. Detailed experimental as well as theoretical investigations have identified hydration, electrostatic, van der Waals or dispersion, and conformational fluctuation forces as the most fundamental interactions governing the fate of biological macromolecules.

Our intent here is to sketch the measurements of these operative forces and to dwell on concepts that rationalize them. It is from these concepts, with their insight into what controls organizing forces, that we expect people to learn to manipulate and package DNA in more rewarding ways.

II. MOLECULAR FORCES

A. The Origin and Measurement of Molecular Forces

We divide these forces into two broad categories, both of which can be either attractive or repulsive. First, there are interactions that are connected with fields emanating from sources within or on the macromolecules themselves (16) (e.g., electrostatic fields pointing from the fixed-charge distributions on macromolecules into the surrounding space, fields of connectivity of hydrogen bond networks extending from the macromolecular surfaces into the bulk solution that are seen in hydration interactions). Second, there are forces due to fluctuations that originate either in thermal Brownian motion or quantum jitter (15). Consequent interactions include the van der Waals or dispersion forces that originate from

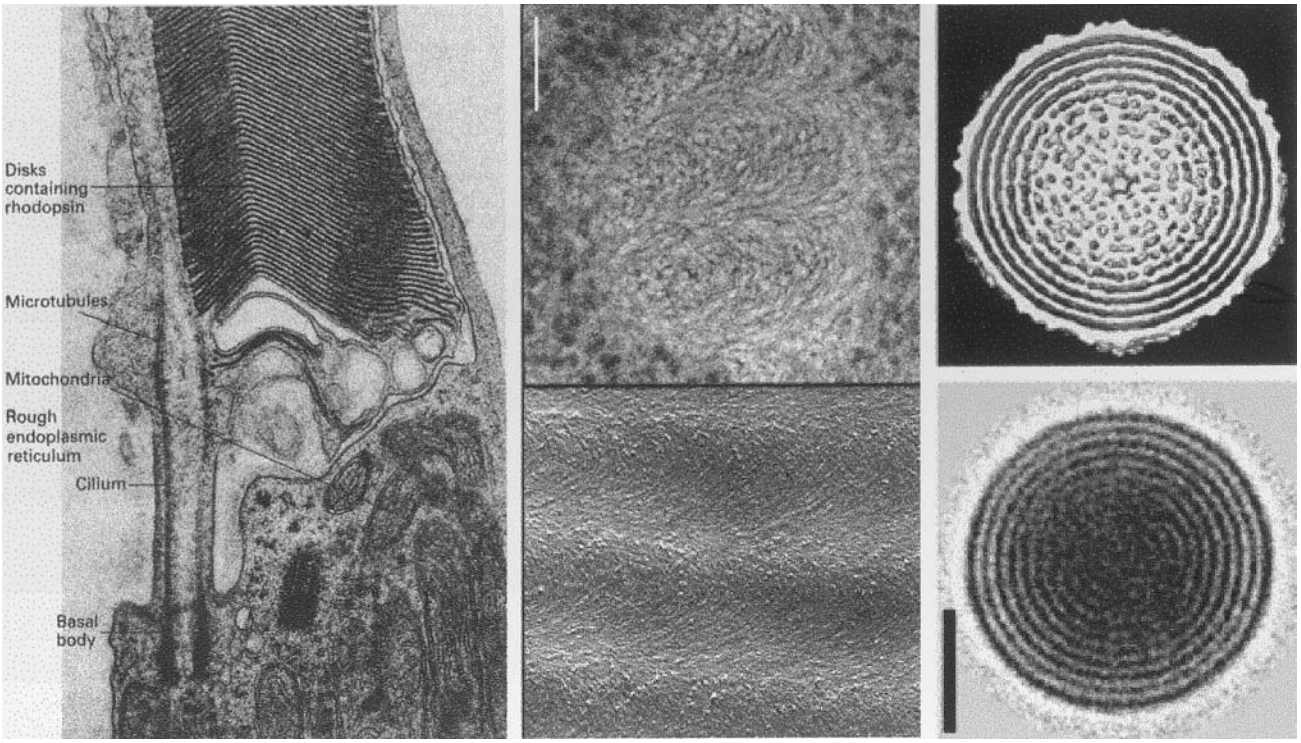


Figure 2 Highly ordered assemblies, ubiquitous among biological structures, can be explained through the properties of a very small number of fundamental forces acting between macromolecules. On the left-hand side, electron micrograph of a part of a human eye rod cell showing multilamellar bilayer aggregate. (From Kessel RG, Kardon RH. Tissues and Organs. San Francisco: W.H. Freeman and Co, 1979.) In the middle, electron micrograph of an in vivo cholesteric phase of a wild-type *E. coli* DNA. (Adapted from D. Frankiel–Krispin et al. EMBO J **20** (2001) 1184–1191.) For comparison we show the same type of structure for DNA in vitro below. (Adapted from A. Leforestier and F. Livolant, Biophys. J **65** (1993) 56–72.) On the right-hand side, cryomicrographs and computer-processed images of T7 phage heads showing ordered DNA spooling within the viral heads. (From Ref. 13.)

thermal as well as quantum mechanical fluctuations of electromagnetic fields in the space between and within the interacting molecules; conformation-fluctuation forces from thermal gyrations by the macromolecule when thermal agitation pushes against the elastic energy resistance of the molecule and confinement imposed by neighboring macromolecules (16).

There are many ways to detect interactions between macromolecules. Here we consider only macromolecules interacting in ordered arrays that are particularly relevant for investigations of the packing and energetics of DNA–lipid complexes.

A fundamental concept in macromolecular arrays is that of osmotic pressure (Fig. 3). It is equal to the pressure needed to hold a macromolecular array together against the forces acting between its constituent macromolecules. It can be applied either mechanically across a semipermeable membrane or via the osmotic stress of a high molecular weight (e.g., PEG (polyethyleneglycol), PVP (polyvinylpyrrolidone), dextrane) polymer solution. At chemical equilibrium, the osmotic pressure of one solution (macromolecular array) balances that of another (the bathing polymer solution). The chemical equilibrium can be maintained either via a semipermeable membrane or simply because the bathing polymer solution phase separates from the macromolecular array, as is many times the case with PEGs, PVP, and dextrane. This osmotical balancing of different molecular solutions is the basis of the ''osmotic stress method'' of measuring the equation of state of macromolecular arrays (18).

The equation of state of a macromolecular solution is defined as the dependence of its osmotic pressure on the density of the array (Fig. 4). By equilibrating the macromolecular array vs. a solution of high molecular weight polymer with a known osmotic pressure, one can set the osmotic pressure in the macromolecular array itself (18). If in addition the concurrent density of the macromolecular array is measured, either via X-ray scattering or direct densitometry, one gets the dependence of the osmotic pressure of the array on its density (i.e., its equation of state). This is the essence of the osmotic stress method.

1. Hydration Force

The hydration force is connected with a simple observation that it takes increasing amounts of work to remove water from

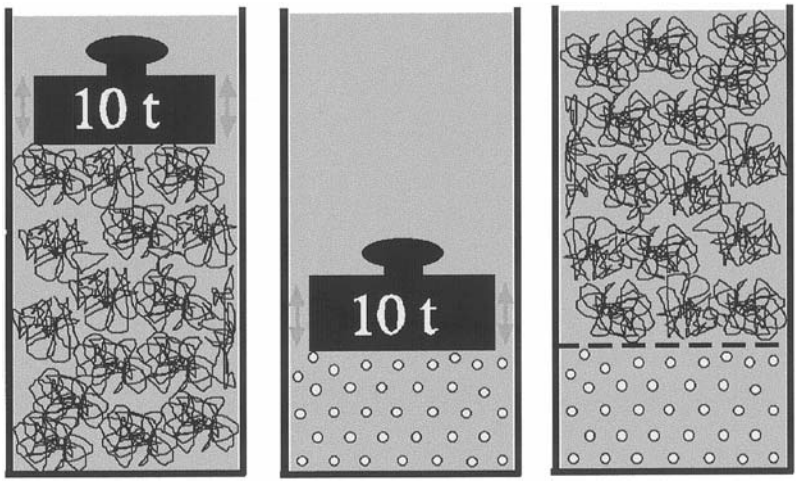


Figure 3 Osmotic pressure in macromolecular arrays. Dissolved polymers such as PEG exert an osmotic pressure on the the part of the solution from which they are excluded (shown schematically by the weight). Instead of exerting directly a pressure on the macromolecular subphase such as DNA or lipid arrays (small circles), one can equilibrate it with a solution of PEG at a set concentration (what amounts to the same thing: a set osmotic pressure) and PEG itself will exert osmotic stress on the macromolecular subphase. Osmotic weighing of polymers one against the other (the one with the known, set osmotic pressure against the unknown one) is the essence of the osmotic stress technique of measuring interactions in macromolecular solutions. See the color insert for a color version of this figure.

between electrically neutral lipids in multilamellar arrays, or from between ordered arrays of polymers at large polymer concentrations (18). Direct measurements of this work strongly suggest that it increases exponentially with the diminishing separation between colloid surfaces with a certain decay length that depends as much on the bulk properties of the solvent as on the detailed characteristics of the interacting surfaces. There is nevertheless some profound universality in the interactions between macromolecular surfaces at close distances (Fig. 5), whether they are charged, zwitterionic, or uncharged, that strongly suggest that water is essential in maintaining the stability of biological matter at high densities.

Hydration forces can be understood in different terms with no consensus yet on mechanism (11). Marčelja and coworkers (19) first proposed the idea that colloid surfaces perturb the vicinal water and that the exponential decay of the hydration force is due to the weakening of the perturbation of the solvent as a function of the distance between the interacting surfaces (Fig. 6). They introduced an order parameter $P(z)$ as a function of the transverse coordinate z, between the surfaces located at $z = D/2$ and $z = -D/2$, that would capture the local condition, or local ordering of solvent molecules between the surfaces. The detailed physical nature of this order parameter is left unspecified, but because the theory builds on general principles of symmetry and perturbation expansions molecular details are not needed. All one needs to know about P is that within the bulk water $P = 0$ and close to a macromolecular surface P remains nonzero. As a mnemonic, one can envision P as an arrow associated with each water molecule. In the bulk, the arrows point in all directions with equal probability. Close to a bounding macromolecular surface, they point preferentially toward or away from the surface (Fig. 6), depending on the surface-orienting fields.

If we envisage solvent molecules between two perturbing surfaces, we can decompose the total free energy F of their configuration into its energy W and entropy S parts via the well-known thermodynamic definition $F = W - TS$, where T is the temperature. Energetically it would be most favorable for the surface-induced order to persist away from the surfaces, but that would create conflict between the apposing surfaces (Fig. 3). Entropy fights any type of ordering and wants to eliminate all orderly configurations between the two surfaces, creating a homogeneous state of molecular disorder characterized by $P = 0$. Energy and entropy compromise to create a nonuniform profile of the order parameter between the surfaces; surface-induced order propagates but progressively decreases away from the surfaces.

From the free energy, we can derive the repulsive hydration osmotic pressure p acting between the surfaces because by definition it is proportional to the derivative of the free energy with respect to the separation D. Osmotic pressure between two apposed lipid surfaces has been measured extensively for different lipids (20) and has been measured to have the form $p = p_0 \exp(-D/\lambda_H)$, consistent with previously theoretically derived form of the hydration free energy if one assumes that $p_0 \sim P^2(z = D/2)$. Here λ_H is the hydration decay length of 0.1–0.4 nm measuring the spatial extent of water perturbation. From these experiments, one can deduce the magnitude of the prefactor p_0, which for a great variety of lipids and lipid mixtures can be found within an interval 10^{12} to 10^{10} dynes/cm^2. This ratio also

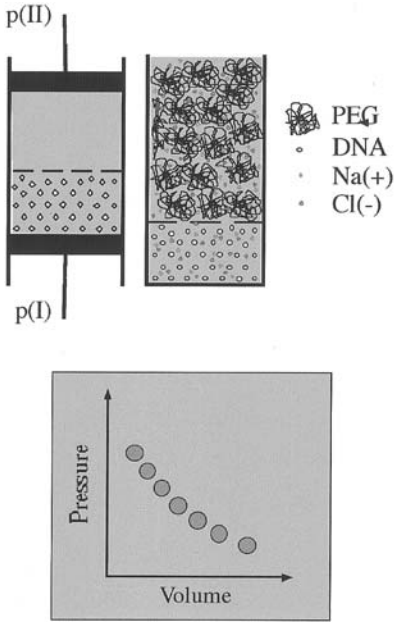


Figure 4 The osmotic stress method (18). DNA liquid crystals are equilibrated against solutions of a neutral polymer (e.g., PEG or PVP, depicted as disordered coils). These solutions are of known osmotic pressure, pH, temperature and ionic composition (54). Equilibration of DNA under the osmotic stress of external polymer solution is effectively the same as exerting mechanical pressure on the DNA subphase with a piston that passes water and small solutes but not DNA. After equilibration under this known stress, DNA separation is measured either by X-ray scattering, if the DNA subphase is sufficiently ordered, or by densitometry (55). DNA density and osmotic stress thus determined immediately provide an equation of state (osmotic pressure as a function of the density of the DNA subphase) to be codified in analytical form over an entire phase diagram. See the color insert for a color version of this figure.

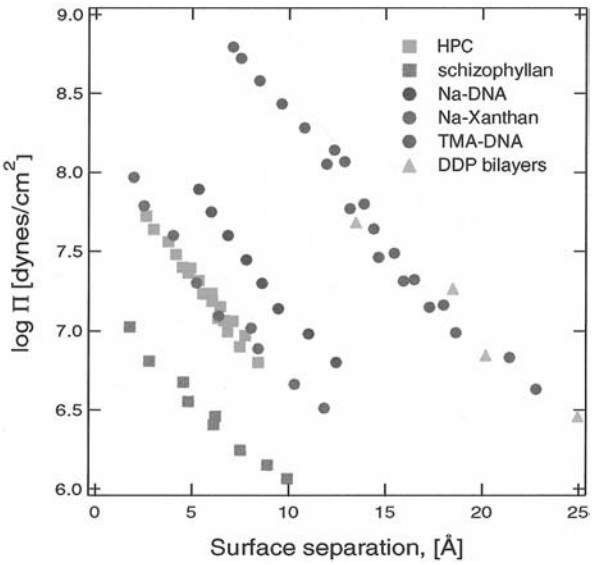


Figure 5 Interactions between biological macromolecules show pronounced universality at close surface-to-surface separations (or equivalently at very large densities). Hydroxypropyl cellulose, schizophillan, different DNA salts, xanthan, and DDP bilayers at small intermolecular separations (given in terms of the separation between effective molecular surfaces of the interacting molecules) all show strong repulsive interactions decaying with about the same characteristic decay length. The log-linear plot is thus more or less a straight line. (Composite data courtesy of D. C. Rau.) See the color insert for a color version of this figure.

determines the absolute magnitude of the hydration repulsion, which can be in the hundreds of atmospheres.

As already noted in this simple theoretical approach, the hydration decay length depends only on the bulk properties of the solvent, and not on the properties of the surface. To generalize this simplification, Kornyshev and Leikin (21) formulated a variant of the hydration force theory to also take into account explicitly the nature of surface ordering. They derive a modified hydration decay length that clearly shows how the surface order couples with the bare hydration decay length. Without going too deeply into this theory, we note that if the interacting surfaces have 2-dimensional ordering patterns characterized by a wave vector $Q = 2/\lambda$, where λ is the characteristic scale of the spatial variations of these patterns, then the effective hydration force decay length would be $\lambda_{KL} = \frac{1}{2}\, l_H\, (1 + 4^2(\lambda_H/\lambda)^2)^{-1/2}$. Inserting numbers for the case of DNA, where the ''surface'' structure has a characteristic scale of 1 to 2 Å, we realize that the hydration decay length in this case would be almost entirely determined by the surface structure and not the bulk solvent properties. Given the experimentally determined variety of forces between phospholipids (20), it is indeed quite possible that even in the simplest cases the measured decay lengths are not only those of the water solvent itself, but also include the surface properties via the characteristic scale of the surface ordering l_H.

The other important facet of this theory is that it predicts that in certain circumstances the hydration forces can become attractive (11). This is particularly important in the case of interacting DNA molecules where this hydration attraction connected with condensing agents can hold DNAs into an ordered array, even though the van der Waals forces themselves would be unable to accomplish that (22). This attraction is always an outcome of nonhomogeneous surface ordering and arises in situations where apposing surfaces have complementary checkerboard-like order (11). Unfortunately, in this situation, many mechanisms can contribute to attractions; therefore, it is difficult to argue for one strongest contribution.

2. Electrostatic Forces

Electrostatic forces between charged colloid bodies are among the key components of the force equilibria in (bio)colloid sys-

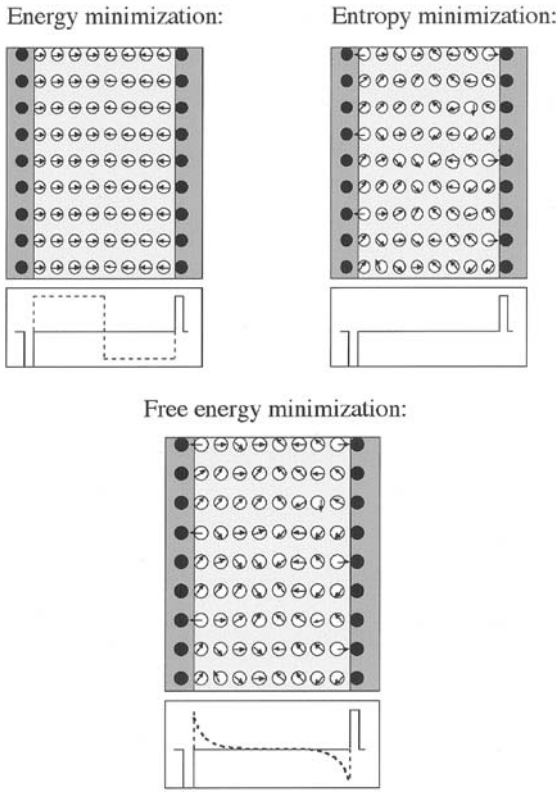


Figure 6 The hydration force. Marcelja and Radic (19) introduced an order parameter P that would capture the local condition, or local ordering, of solvent molecules between the surfaces. We represent it as an arrow (that has magnitude and direction) on each water molecule that is trapped between the two apposing surfaces and is being acted upon by the surface fields, depicted schematically with a bold line below each of the three drawings. Minimizing the energy corresponding to a spatial profile of P, leads to a configuration where P points (for example) away from both surfaces, and there is thus mismatch at the midplane (the dotted line below the leftmost drawing). The entropy would favor completely disordered configurations with no net value of P (the dotted line below the rightmost drawing). The free energy strikes a compromise between the two extrema, leading to a smooth profile of P, varying continuously as one goes from one surface to the other (the dotted line below the bottom drawing). As the two surfaces approach the nonmonotonic profile of the order parameter P leads to repulsive forces between them. See the color insert for a color version of this figure.

tems (23). At larger separations, they are the only forces that can counteract van der Waals attractions and thus stabilize colloid assembly. The crucial role of the electrostatic interactions in (bio)colloid systems is well documented and explored, following the seminal realization of Bernal and Fankuchen (24) that electrostatic interaction is the stabilizing force in tobacco mosaic virus (TMV) arrays.

Although the salient features of electrostatic interactions of fixed charges in a sea of mobile countercharges and salt ions are intuitively straightforward to understand, they are difficult to evaluate. These difficulties are clearly displayed by the early ambiguities in the sign of electrostatic interactions between two equally charged bodies that were first claimed to be attractive (Levine), then repulsive (Verwey-Overbeek), and finally that they were usually repulsive except if the counterions or the salt ions are of higher valency (25).

Here we introduce the electrostatic interaction on an intuitive footing (Fig. 7). Assume we have two equally charged bodies with counterions in-between. Clearly the minimum of electrostatic energy W_E (28), which for the electrostatic field configuration at the spatial position r, $E(r)$, is proportional to the integral of $E^2(r)$ over the whole space where one has nonzero electrostatic field, would correspond to adsorption of counterions to the charges leading to their complete neutralization. The equilibrium electrostatic field would thus be entirely concentrated next to the surface. However, at finite temperatures, it is not the electrostatic energy but rather the free energy (26), $F = W_E - TS$, also containing the entropy S of the counterion distribution, that should be minimized. The entropy of the mobile particles with the local density $\rho_i(r)$ [we assume there are more than 1 species of mobile particles, (e.g., counterions and salt ions) tracked through the index i] is taken as an ideal gas entropy (26), which is proportional to the volume integral of $\Sigma_i[\rho_i(r)\ln(\rho_i(r)/_{i0}) - (\rho_i(r)\text{-}\rho_{i0})]$, where ρ_{i0} is the density of the mobile charges in a reservoir that is in chemical equilibrium with the confined system under investigation. Entropy by itself would clearly lead to a uniform distribution of counterions between the charged bodies, $\rho_i(r) = \rho_{i0}$, whereas together with the electrostatic energy it obviously leads to a nonmonotonic profile of the mobile charge distribution between the surfaces, minimizing the total free energy of the mobile ions.

The above discussion, although far from being rigorous, contains the important theoretical underpinnings known as the Poisson–Boltzmann theory (27). To arrive at the central equation corresponding to the core of this theory, one simply has to formally minimize the free energy $F = W_E - TS$, just as in the case of structural interactions, together with the basic electrostatic equation (28) (the Poisson equation) that connects the sources of the electrostatic field with the charge densities of different ionic species. The standard procedure is now to minimize the free energy, take into account the Poisson equation, and what follows is the well-known Poisson–Boltzmann equation, the solution of which gives the nonuniform profile of the mobile charges between the surfaces with fixed charges. This equation can be solved explicitly for some particularly simple geometries (27). For two charged planar surfaces, the solution gives a screened electrostatic potential that decays exponentially away from the walls. It is thus smallest in the middle of the region between the surfaces and largest at the surfaces. The spatial variation of the electrostatic interaction is just as in the case of structural interactions described with a characteristic decay length, dubbed the Debye length in this case, which for uni-uni valent salts assumes the value

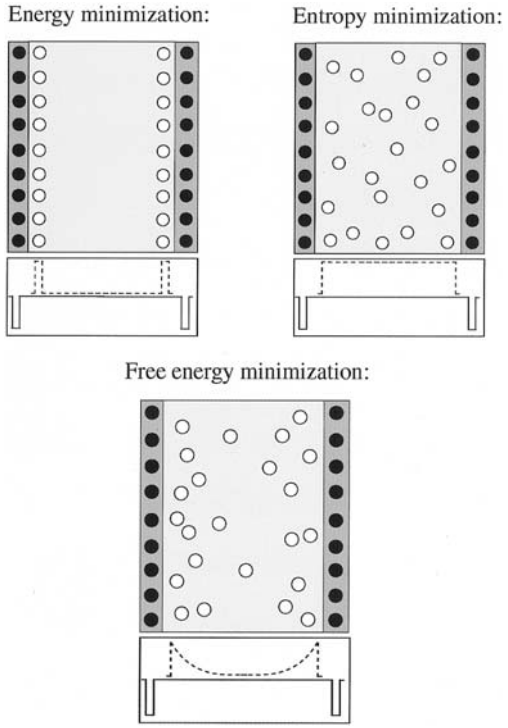


Figure 7 A pictorial exposition of the main ideas behind the Poisson–Boltzmann theory of electrostatic interactions between (bio)colloidal surfaces. Electrostatic energy by itself would favor adsorption of counterions (white circles) to the oppositely charged surfaces (black circles). The equilibrium profile of the counterions in this case is presented by the dotted line below the leftmost drawing. Entropy, to the contrary, favors a completely disordered configuration (i.e., a uniform distribution of counterions between the surfaces), presented by the dotted line below the rightmost drawing. The free energy works a compromise between the two principles leading to a nonmonotonic profile of the counterion density (25), varying smoothly in the intersurface region. As the two surfaces are brought close, the overlapping counterion distributions originating at the fixed charge at the surfaces (the bold line below each drawing) create repulsive forces between them. See the color insert for a color version of this figure.

of $\lambda_D = 3$ Å$/\sqrt{I}$, where I is the ionic strength of the salt in moles per liter. A 0.1 molar solution of uni-uni valent salt, such as NaCl, would thus have the characteristic decay length of about 9.5 Å. Beyond this separation, the charged bodies no longer feel each other. By adding or removing salt from the bathing solution, we are thus able to regulate the range of electrostatic interactions.

The exponential decay of the electrostatic field away from the charged surfaces with a characteristic length, independent (to the lowest order) of the surface charge, is one of the most important results of the Poisson–Boltzmann theory.

Obviously, as the surfaces come closer together, their decaying electrostatic potentials begin to interpenetrate (25). The consequence of this interpenetration is a repulsive force between the surfaces that again decays exponentially with the intersurface separation and a characteristic length again equal to the Debye length. For two planar surfaces at a separation D, bearing sufficiently small charges, characterized by the surface charge density σ, so that the ensuing electrostatic potential is never larger than k_BT/e, where k_B is Boltzmann's constant and e is the elementary electron charge, one can derive (27) for the interaction free energy per unit surface area $F(D)$ the expression $F(D) \sim \sigma^2 \exp(-D/\lambda_D)$. Obviously the typical magnitude of the electrostatic interaction in different systems depends on the magnitude of the surface charge. It would not be unusual in lipids to have surface charge densities in the range of 1 elementary charge per 50 to 100 Å^2 surface area (29). For this range of surface charge densitities, the constant prefactor in the expression for the osmotic pressure would be of the order 0.4 to 1.2×10^7 N/m.

The same type of analysis would also apply to two charged cylindrical bodies (e.g., two molecules of DNA) interacting across an electrolyte solution. What one evaluates in this case is the interaction free energy per unit length of the cylinders (30), $g(R)$, where R is the separation between the cylinders that can be obtained in the approximate form $g(R) \sim \mu^2 \exp(-R/\lambda_D)$. It is actually possible to also get an explicit form (30) of the interaction energy between two cylinders even if they are skewed by an angle θ between them. In this case, the relevant quantity is the interaction free energy itself (if θ is nonzero, then the interaction energy does not scale with the length of the molecules) that can be obtained in a closed form as $F(R,\theta) \sim \mu^2 \lambda_D R^{1/2} \exp(-R/\lambda_D)/\sin(\theta)$.

The predictions for the forces between charged colloid bodies have been reasonably well borne out for electrolyte solutions of uni-uni valent salts (31). In that case, there is near quantitative agreement between theory and experiment. However, for higher valency salts, the Poisson–Boltzmann theory does not only give the wrong numerical values for the strength of the electrostatic interactions, but also misses their sign. In higher valency salts, the correlations among mobile charges between charged colloid bodies due to thermal fluctuations in their mean concentration lead effectively to attractive interactions (32) that are in many respects similar to the van der Waals forces that are analyzed next.

3. van der Waals Forces

van der Waals charge fluctuation forces are special in the sense that they are a consequence of thermodynamic and quantum mechanical fluctuations of the electromagnetic fields (15). They exist even if the average charge, dipole moment, or higher multipole moments on the colloid bodies are zero. This is in stark contrast to electrostatic forces that require a net charge or a net polarization to drive the interaction. This also signifies that the van der Waals forces are much more general and ubiquitous than any other force between colloid bodies (9).

There are many different approaches to the van der Waals forces (15,33). For interacting molecules, one can distinguish different contributions to the van der Waals force, stemming from thermally averaged dipole–dipole potentials (the Keesom interaction), dipole-induced dipole interactions (the Debye interaction), and induced dipole-induced dipole interactions (the London interaction) (34). They are all attractive and their respective interaction energy decays as the sixth power of the separation between the interacting molecules. The magnitude of the interaction energy depends on the electromagnetic absorption (dispersion) spectrum of interacting bodies, thus also the term dispersion forces.

For large colloidal bodies composed of many molecules, the calculation of the total van der Waals interactions is no trivial matter (15), even if we know the interactions between individual molecules composing the bodies. Hamaker assumed that one can simply add the interactions between composing molecules in a pairwise manner. It turned out that this was a very crude and simplistic approach to van der Waals forces in colloidal systems because it does not take into account the highly nonlinear nature of the van der Waals interactions in condensed media. Molecules in a condensed body interact among themselves, thus changing their properties (c.f. their dispersion spectrum) that in their turn modify the van der Waals forces between them.

Lifshitz, following work of Casimir (9,15), realized how to circumvent this difficulty and formulated the theory of van der Waals forces in a way that already includes all these nonlinearities. The main assumption of this theory is that the presence of dielectric discontinuities as in colloid surfaces, modifies the spectrum of electromagnetic field modes between these surfaces (Fig. 8). As the separation between colloid bodies varies, so do the eigenmode frequencies of the electromagnetic field between and within the colloid bodies. It is possible to deduce the change in the free energy of the electromagnetic modes due to the changes in the separation between colloid bodies coupled to their dispersion spectral characteristics (35).

Based on the work of Lifshitz, it is now clear that the van der Waals interaction energy is just the change of the free energy of field harmonic oscillators at a particular eigenmode frequency ω as a function of the separation between the interacting bodies D and temperature T, $\omega = \omega(D,T)$. With this equivalence in mind, it is quite straightforward to calculate the van der Waals interaction free energy between two planar surfaces at a separation D and temperature T; the dielectric permittivity between the two surfaces, ε and within the surfaces, ε', must both be known as a function of the frequency of the electromagnetic field (35). This is a consequence of the fact that, in general, the dielectric media comprising the surfaces as well as the space between them are dispersive, meaning that their dielectric permittivities depend on frequency of the electromagnetic field [i.e., $\varepsilon = \varepsilon(\omega)$]. With this in mind one can derive the interaction free energy per unit surface area of the interacting surfaces in the form $F(D) = A/12\pi D^2$, where the s.c. Hamaker coefficient A depends on the difference between the dielectric permittivities of the interacting materials at different imaginary frequencies. It can be in general split into two terms: the first term in the Hamaker coefficient is due to thermodynamic fluctuations, such as Brownian rotations of the dipoles of the molecules composing the media or the averaged dipole-induced dipole forces and depends on the static ($\omega = 0$) dielectric response of the interacting media, whereas the second term is purely quantum mechanical in nature (15). The imaginary argument of the dielectric constants is not that odd because $\varepsilon(\iota\zeta)$ is an even function of ζ, which makes $\varepsilon(\iota\zeta)$ also a purely real quantity (35).

To evaluate the magnitude of the van der Waals forces, one thus has to know the dielectric dispersion $\varepsilon(\omega)$ of all the media involved. This is no simple task and can be accomplished only for very few materials (34). Experiments seem to be a much more straightforward way to proceed. The values for the Hamaker coefficients of different materials interacting

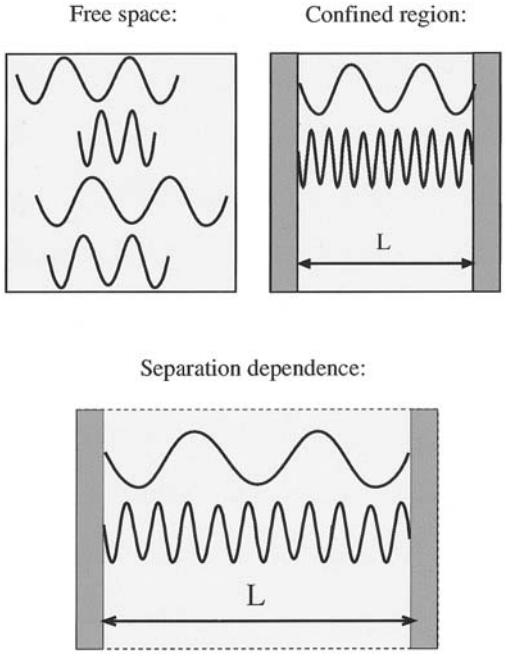


Figure 8 A pictorial introduction to the theory of Lifshitz–van der Waals forces between colloid bodies. Empty space is alive with electromagnetic (EM) field modes that are excited by thermal as well as quantum mechanical fluctuations. Their frequency is unconstrained and follows the black body radiation law. Between dielectric bodies, only those EM modes survive that can fit into a confined geometry. As the width of the space between the bodies varies, so do the allowed EM mode frequencies. Every mode can be treated as a separate harmonic oscillator, each contributing to the free energy of the system. Because this free energy depends on the frequency of the modes, that in turn depend on the separation between the bodies, the total free energy of the EM modes depends on the separation between the bodies. This is an intuitive description of the Lifshitz–van der Waals force (15). See the color insert for a color version of this figure.

across water are between 0.3 and 2.0 $\times$ 10^{-20} J. Specifically for lipids, the Hamaker constants are quite close to theoretical expectations except for the phosphatidylethanolamines that show much larger attractive interactions probably due to headgroup alignment (31). Evidence from direct measurements of attractive contact energies as well as direct force measurements suggest that van der Waals forces are more than adequate to provide attraction between bilayers for them to form multilamellar systems (36).

For cylinders the same type of argument applies, except that due to the geometry the calculations are a bit more tedious (37). Here the relevant quantity is not the free energy per unit area but the interaction free energy per unit length of the two cylinders of radius a, $g(R)$, considered to be parallel at a separation R. The calculation (38) leads to the following form $g(R) \sim A\ a^4/R^5$, where the constant A again depends on the differences between dielectric permittivities $\varepsilon_{\parallel}$, the parallel, and $\varepsilon_{\perp}$, the perpendicular components of the dielectric permittivity of the dielectric material of the cylinders, and ε_m, the dielectric permittivity of the bathing medium.

If, however, the 2 interacting cylinders are skewed, then the interaction free energy $G(R, \theta)$, this time not per length, is obtained (38) in the form $G(R) \sim (A + B\cos^2\theta)(a^4/R^4\sin\theta.)$ The constants A and B describe the dielectric mismatch between the cylinder and the bathing medium at different imaginary frequencies. The same correspondence between the thermodynamic and quantum mechanical parts of the interactions as for two parallel cylinders also applies to this case. Clearly, the van der Waals force between two cylinders has a profound angular dependence that in general creates torques between the two interacting molecules.

Taking the numerical values of the dielectric permittivities for two interacting DNA molecules, one can calculate that the van der Waals forces are quite small, typically 1 to 2 orders of magnitude smaller than the electrostatic repulsions between them, and in general cannot hold the DNAs together in an ordered array. Other forces, leading to condensation phenomena in DNA (10) clearly have to be added to the total force balance in order to get a stable array. There is as yet still no consensus on the exact nature of these additional attractions. It seems that they are due to the fluctuations of counterion atmosphere close to the molecules.

4. The DLVO Model

The popular Derjaguin-Landau-Verwey-Overbeek (DLVO) (9,25) model assumes that electrostatic double-layer and van der Waals interactions govern colloid stability. Applied with a piety not anticipated by its founders, this model actually does work rather well in surprisingly many cases. Direct osmotic stress measurements of forces between lipid bilayers show that at separations less than $\sim$10 Å there are qualitative deviations from DLVO thinking (39). For micron-size objects and for macromolecules at greater separations, electrostatic double-layer forces and sometimes van der Waals forces tell us what we need to know about interactions governing movement and packing.

5. Geometric Effects

Forces between macromolecular surfaces are most easily analyzed in plane parallel geometry. Because most of the interacting colloid surfaces are not planar, one has either to evaluate molecular interactions for each particular geometry or to devise a way to connect the forces between planar surfaces with forces between surfaces of a more general shape. The Derjaguin approximation (9) assumes that interactions between curved bodies can be decomposed into interactions between small plane-parallel sections of the curved bodies (Fig. 9). The total interaction between curved bodies would be thus equal to a sum where each term corresponds to a partial interaction between quasi-plane-parallel sections of the two bodies. This idea can be given a completely rigorous form and leads to a connection between the interaction free energy per unit area of two interacting planar surfaces, $F(D)$, and the force acting between two spheres at minimal separation D, $f(D)$, 1 with the mean radius of curvature R_1 and the other 1 with R_2. The formal equivalence can be written as follows, $f(D) = 2\pi (R_1R_2/(R_1 + R_2))\ F(D)$. A similar equation can also be obtained for 2 cylinders in the form, $f(D) = 2\pi(R_1R_2)^{1/2}\ F(D)$.

These approximate relations clearly make the problem of calculating interactions between bodies of general shape tractable. The only caveat here is that the radii of curvature should be much larger than the proximal separation between the two interacting bodies, effectively limiting the Derjaguin approximation to sufficiently small separations.

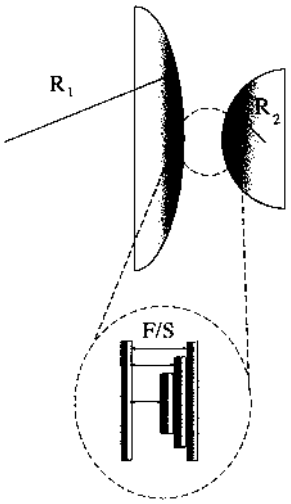


Figure 9 The Derjaguin approximation. To formulate forces between oppositely curved bodies (e.g., cylinders, spheres, etc.) is very difficult, but it is often possible to use an approximate procedure. Two curved bodies (two spheres of unequal radii in this case) are approximated by a succession of planar sections, interactions between which can be calculated relatively easy. The total interaction between curved bodies is obtained through a summation over these planar sections.

Using the Derjaguin formula or evaluating the interaction energy explicitly for those geometries for which this indeed is not an insurmountable task, one can now obtain a whole zoo of DLVO expressions for different interaction geometries (Fig. 10). The salient features of all these expressions are that the total interaction free energy always has a primary minimum, that can only be eliminated by strong short-range hydration forces, and a secondary minimum due to the compensation of screened electrostatic repulsion and van der Waals–Lifshitz attraction. The position of the secondary minimum depends as much on the parameters of the forces (Hamaker constant, fixed charges, and ionic strength) as on the interaction geometry. Generally, the range of interaction between the bodies of different shapes is inversely proportional to their radii of curvature.

Thus, the longest-range forces are observed between planar bodies, and the shortest between small (pointlike) bodies.

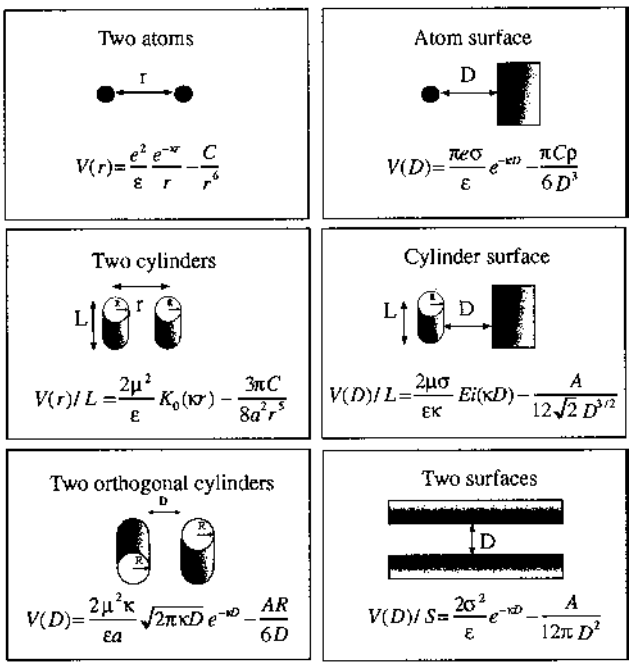


Figure 10 A menagerie of DLVO interaction expressions for different geometries most commonly encountered in biological milieu. Two small particles, a particle and a wall, 2 parallel cylinders, a cylinder close to a wall, 2 skewed cylinders and 2 walls. The DLVO interaction free energy is always composed of a repulsive electrostatic part (calculated from a linearized Poisson–Boltzmann theory) and an attractive van der Waals part. Charge: e, charge per unit length of a cylinder: μ, charge per unit surface area of a wall: σ, C is a geometry-dependent constant, ε the dielectric constant, κ the inverse Debye length, and ρ the density of the wall material. The functions $K_0(x)$ (the Bessel function K_0) and $Ei(x)$ (the exponential integral function) both depend essentially exponentially on their respective argument.

What we have not indicated in Fig. 7 is that the interaction energy between two cylindrical bodies, skewed at a general angle θ and not just for parallel or crossed configurations, can be obtained in an explicit form. It follows simply from these results that the configuration of two interacting rods with minimal interaction energy is the one corresponding to $\theta=\pi/2$ (i.e., corresponding to crossed rods).

6. Fluctuation Forces

The term ''fluctuation forces'' is a bit misleading in this context because clearly van der Waals forces are already fluctuation forces. What we have in mind is thus a generalization of the van der Waals forces to situations where the fluctuating quantities are not electromagnetic fields but other quantities subject to thermal fluctuations. No general observation as to the sign of these interactions can be made, they can be either repulsive or attractive and are as a rule of thumb comparable in magnitude to the van der Waals forces.

The most important and ubiquitous force in this category is the undulation or Helfrich force (41). It has a very simple origin and operates among any type of deformable bodies as long as their curvature moduli are small enough (comparable to thermal energies). It was shown to be important for multilamellar lipid arrays (41) as well as in hexagonal polyelectrolyte arrays (42) (Fig. 11).

The mechanism is simple. The shape of deformable bodies fluctuates because of thermal agitation (Brownian motion) (26). If the bodies are close to each other, the conformational fluctuations of one will be constrained by the fluctuations of its neighbors. Thermal motion makes the bodies bump into each other, which creates spikes of repulsive force between them. The average of this force is smooth and decays continuously with the mean separation between the bodies.

One can estimate this steric interaction for multilamellar lipid systems and for condensed arrays of cylindrical polymers (Fig. 11). The only quantity entering this calculation is the elastic energy of a single bilayer that can be written as the square of the average curvature of the surface, summed over the whole area of the surface, multiplied by the elastic modulus of the membrane, K_C. K_C is usually between 10 and 50 k_BT (43) for different lipid membranes. If the instantaneous deviation of the membrane from its overall planar shape in the plane is now introduced as u, the presence of neighboring membranes introduces a constraint on the fluctuations of u that basically demands, that the average of the square of u must be proportional to D^2, where D is the average separation between the membranes in a multilamellar stack. Thus, we should have $u^2 \sim D^2$. The free energy associated with this constraint can now be derived in the form (40) $F(D) \sim (k_BT)^2/(K_CD^2)$, and is seen to decay in inverse proportion to the separation between bilayers squared.

It has thus obviously the same dependence on D as the van der Waals force. This is, however, not a general feature of undulation interactions as the next example clearly shows. Also, we only indicated the general proportionality of the interaction energy. Calculation of the prefactors can be a difficult (44), especially because the elastic bodies usually do not

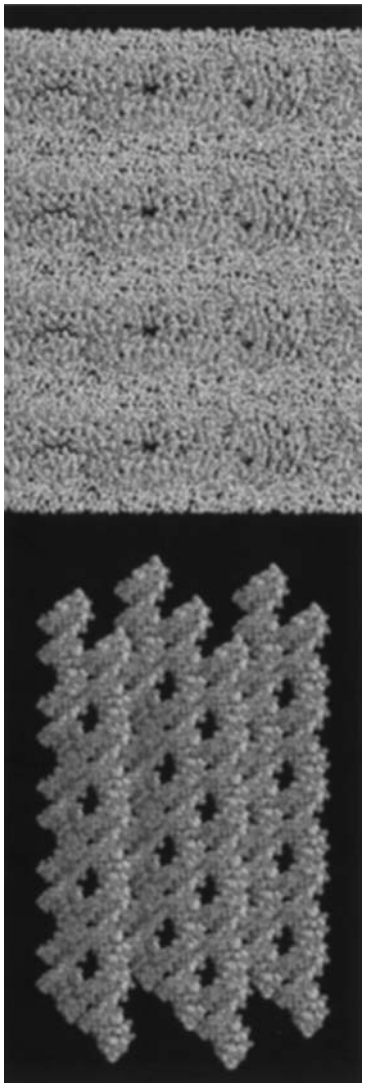


Figure 11 Thermally excited conformational fluctuations in a multilamellar membrane array (small molecules are waters and long-chain molecules are phospholipids) or in a tightly packed polyelectrolyte chain array (the figure represents a hexagonally packed DNA array) lead to collisions between membranes or polyelectrolyte chains. These collisions contribute an additional repulsive contribution to the total osmotic pressure in the array, a repulsion that depends on the average spacing between the fluctuating objects. See the color insert for a color version of this figure.

interact with idealized hard repulsions but rather through soft potentials that have both attractive as well as repulsive regimes.

The same line of thought can now be applied to flexible polymers in a condensed array (42). This system is a 1-dimensional analog of the multilamellar membrane system. For polymers, the elastic energy can be written similarly to the membrane case as the square of the local curvature of the polymer, multiplied by the elastic modulus of the polymer, integrated over its whole length. The elastic modulus K_c is usually expressed through a persistence length $L_p = K_c/(k_BT)$. The value of the persistence length tells us how long a polymer can be before the thermal motion forces it to fluctuate wildly. For DNA, this length is about 50 nm. However, it spans the whole range of values between about 10 nm for hyaluronic acid, all the way to 3 mm for microtubules. Using the same constraint for the average fluctuations of the polymer away from the straight axis, one derives for the free energy change due to this constraint the relationship $F(D) \sim (k_BT)/(L_p{}^{1/3}D^{2/3})$ (42).

Clearly, the D dependence for this geometry is much different from the one for van der Waals force, which would be D^{-5}. There is thus no general connection between the van der Waals force and the undulation fluctuation force. Here again, one has to indicate that if the interaction potential between fluctuating bodies is described by a soft potential, with no discernible hard core, the fluctuation interaction can have a profoundly different dependence on the mean separation (42).

Apart from the undulation fluctuation force, there are other fluctuation forces. The most important among them appears to be the monopolar charge fluctuation force (45), recently investigated in the context of DNA condensation. It arises from transient charge fluctuations along the DNA molecule due to constant statistical redistributions of the counterion atmosphere.

The theory of charge fluctuation forces is quite intricate and mathematically demanding (46). Let us just quote a rather interesting result, viz. if two point charges interact via a ''bare'' potential $V_0(R)$, where R is the separation between them, then the effect of the thermal fluctuations in the number of counterions surrounding these charges would lead to an effective interaction of the form $V(R) \sim - k_BTV_0{}^2(R)$. The fluctuation interaction in this case would thus be attractive and proportional to the square of the bare interaction.

This simple result already shows one of the salient features of the interaction potential for monopolar charge fluctuation forces, viz. it is screened with half the Debye screening length [because of $V_0{}^2(R)$]. If there is no screening, however, the monopolar charge fluctuation force becomes the strongest and longest ranged among all fluctuation forces. It is however much less general than the related van der Waals force, and it is still not clear what the detailed conditions should be for its appearance, the main difficulty being the question of whether charge fluctuations in the counterion atmosphere are constrained.

7. Lessons

Molecular forces apparently convey a variety that is surprising considering that they are all to some extent or another just a variant of electrostatic interactions. Quantum and thermal fluctuations apparently modify the underlying electrostatics, leading to qualitatively novel and unexpected features. The

zoo of forces obtained in this way is what one has to deal with and understand when trying to make them work for us.

III. DNA MESOPHASES

A. Polyelectrolyte Properties of DNA

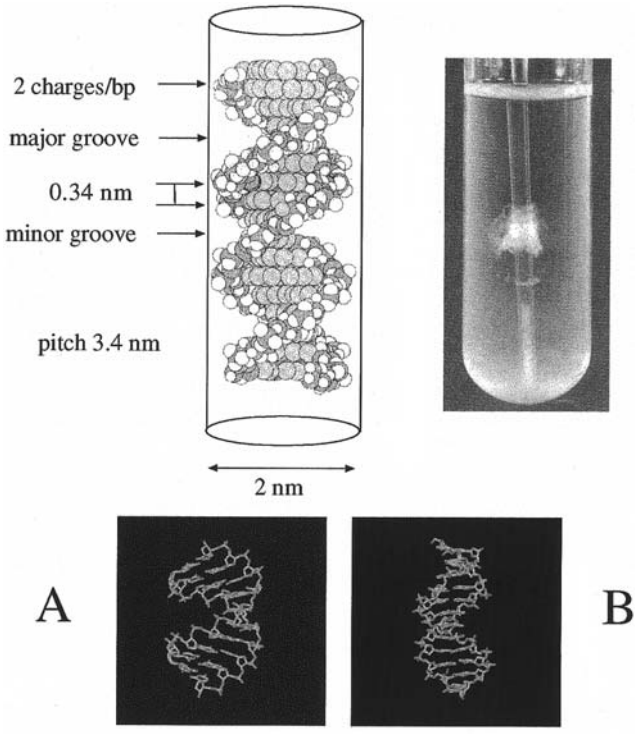
We can define several levels of DNA organization similarly to (1). Its primary structure is the sequence of base pairs. Its secondary structure is the famous double helix that can exist in several conformations. In solution, the B-helical structure dominates (47). The bases are perpendicular to the axis of the molecule and are 0.34 nm apart, and 10 of them make 1 turn of the helix. These parameters can vary for DNA in solution where up to 10.5 base pairs can make a whole turn of the double helix (48). In the A structure, the bases are tilted with respect to the direction of the helix, and this arrangement yields an internal hole, wider diameter, and closer packing (Fig. 12). Other conformations, such as the left-handed Z form, are rare. In solution, DNA's tertiary structure includes the many bent and twisted conformations in 3 dimensions.

DNA lengths can reach macroscopic dimensions. For instance, the human genome is coded in approximately 3 billion base pairs with a collective linear stretch on the order of a meter. Obviously, this molecule must undergo extensive compaction in order to fit in the cell nucleus. In natural environments, DNA is packaged by basic proteins, which form chromatin structures to keep DNA organized. In the test tube, DNA can be packaged into very tight and dense structures as well, primarily by various ''condensing'' agents. Their addition typically induces a random coil to globule transition. At large concentrations, DNA molecules, like lipids, form ordered liquid crystalline phases (10) that have been studied extensively at different solution conditions (8).

In vitro, at concentrations above a critical value (49), polyelectrolyte DNA self-organizes in highly ordered mesophases (8). In this respect, it is a lyotropic liquid crystal. But contrary to the case of lipid mesophases, where the shape of constituent molecules plays a determining role, the organization of DNA in condensed phases is primarily a consequence of its relatively large stiffness (8). The orientational ordering of DNA at high concentrations is promoted mostly by the interplay between entropically favored disorder or misalignment and the consequent price in terms of the high interaction energy. The mechanism of orientational ordering is thus the same as in standard short nematogens (50). The main difference being due to the large length of polymeric chains. The discussion that follows concentrates mostly on very long, on the order of 1000 persistence lengths thus microns long, DNA molecules.

B. Flexibility of DNA Molecules in Solution

In isotropic solutions, DNA can be in one of several forms. For linear DNA, individual molecules are effectively straight over the span of a persistence length that can also be defined as the exponential decay length for the loss of angular correlation between 2 positions along the molecule, while for longer



Polymer	Solvent	Persistence length [nm]
Microtubule	water	3000000
F-actin	water	15000
Schizophyllan	water	200
Xanthan	0.1 M NaCl	120
DNA	0.2 M NaCl	50
Hyaluronic acid	0.2 M NaCl	7

Figure 12 Structural parameters of a DNA molecule. The two relevant configurations of the DNA backbone: A-DNA, common at small hydrations or high DNA densities, and B-DNA common in solution at large hydrations and lower DNA densities. The test tube holds ethanol-precipitated DNA in solution. Its milky color is due to the light scattering by thermal conformational fluctuations in the hexatic phase (see main text). Box: typical persistence lengths for different (bio)polymer chains in nm. See the color insert for a color version of this figure.

lengths they form a wormlike random coil. The persistence length of DNA is about 50 nm (1). The persistence length has been determined by measuring the diffusion coefficient of different-length DNA molecules using dynamic light scattering and by enzymatic cyclization reactions (51). It depends only weakly on the base-pair sequence and ionic strength.

DNA can also be circular as in the case of a plasmid. The closed form of a plasmid introduces an additional topological constraint on the conformation that is given by the linking number Lk (2). The linking number gives the number of heli-

cal turns along a circular DNA molecule. Because plasmid DNA is closed, Lk has to be an integer number. By convention, Lk of a closed right-handed DNA helix is positive. The most frequent DNA conformation for plasmids in cells is negatively supercoiled. This means that for such plasmids Lk is less than it would be for a torsionally relaxed DNA circle—negatively supercoiled DNA is underwound. This is a general phenomenon with important biological consequences. It seems that free energy of negative supercoiling catalyzes processes that depend on DNA untwisting, such as DNA replication and transcription, which rely on DNA (52). Although the sequence of bases in exons determine the nature of proteins synthesized, it is possible that such structural features dictate the temporal and spatial evolution of DNA-encoded information.

C. Liquid Crystals

The fact that DNA is intrinsically stiff makes it form liquid crystals at high concentration (8). Known for about 100 years, the simplest liquid crystals are formed by rodlike molecules. Solutions of rods exhibit a transition from an isotropic phase with no preferential orientation to a nematic phase, a fluid in which the axes of all molecules point on average in 1 direction (Fig. 11). The unit vector in which the molecules point is called the nematic director n. Nematic order is orientational order (50), in contrast to positional order that distinguishes between fluid and crystalline phases. Polymers with intrinsic stiffness can also form liquid crystals. This is because a long polymer with persistence length L_p acts much like a solution of individual rods that are all one persistence length long, thus the term ''polymer nematics'' (53).

If the molecules that comprise the liquid crystal are chiral, have a natural twist such as double-helical DNA, then their orientational order tends to twist. This twist originates from the interaction between two molecules that are both of the same handedness. This chiral interaction is illustrated in Fig. 13 for two helical or screwlike molecules. For steric reasons, two helices pack best when tilted with respect to each other. Instead of a nematic phase chiral molecules form a cholesteric phase (50). The cholesteric phase is a twisted nematic phase in which the nematic director twists continuously around the so-called cholesteric axis as shown in Fig. 13. Using the same arguments as for plain polymers, chiral polymers will form polymer cholesterics.

Both cholesteric and hexagonal liquid crystalline DNA phases were identified in the 1960s. This discovery was especially exciting because both phases were also found in biological systems. The hexagonal liquid crystalline phase can be seen in bacterial phages, and the cholesteric phase can be seen in cell nuclei of dinoflagellates (8).

D. Measurements of Forces Between DNA Molecules

Liquid crystalline order lets us measure intermolecular forces directly. With the osmotic stress method, DNA liquid crystals

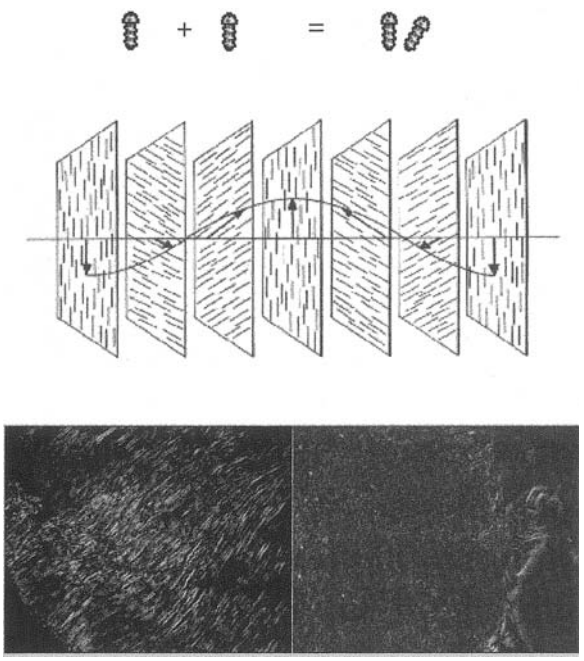


Figure 13 Chiral interaction for two helical or screwlike molecules. For steric reasons, two helices just as two screws (depicted on the figure) pack best when slightly tilted with respect to each other. Because of DNA's double-stranded, helical nature, it is a type of molecular screw and exhibits chiral interactions. Instead of a nematic phase depicted on Fig. 11, characterized by the average constant direction of molecules, chiral molecules form a cholesteric phase (50). The cholesteric phase is a twisted nematic phase in which the nematic director twists continuously around a ''cholesteric axis'' depicted on the middle drawing. Under crossed polarizers (bottom), the DNA cholesteric phase creates a characteristic striated texture. For long DNA molecules, the striations appear disordered.

are equilibrated against neutral polymer (e.g., PEG or PVP) solutions of known osmotic pressure, pH, temperature, and ionic composition (54). Equilibration of DNA under osmotic stress of external polymer solution is effectively the same as exerting mechanical pressure on the DNA subphase with a piston [see Fig. 4]. In this respect, the osmotic stress technique is formally much similar to the Boyle experiment where one compresses a gas with mechanical pistons and measures the ensuing pressure. After equilibration under this known stress, DNA separation is measured either by X-ray scattering, if the DNA subphase is sufficiently ordered, or by straightforward densitometry (55). Known DNA density and osmotic stress immediately provide an equation of state (osmotic pressure as a function of the density of the DNA subphase) to be codified in analytical form for the entire phase diagram. Then, with the local packing symmetry derived from X-ray scatter-

ing (7,54), and sometimes to correct for DNA motion (42), it is possible to extract the bare interaxial forces between molecules that can be compared with theoretical predictions as developed in Chapter 2. In vivo observation of DNA liquid crystals (56) shows that the amount of stress needed for compaction and liquid crystalline ordering is the same as for DNA in vitro.

E. Interactions Between DNA Molecules

Performed on DNA in univalent salt solutions, direct force measurements reveal two types of purely repulsive interactions between DNA double helices (4):

1. At interaxial separations less than ~3 nm (surface separation ~1 nm), an exponentially varying ''hydration'' repulsion believed to originate from partially ordered water near the DNA surface.
2. At surface separations greater than 1 nm, measured interactions reveal electrostatic double-layer repulsion, presumably from negative phosphates along the DNA backbone.

Measurements give no evidence for a significant DNA–DNA attraction expected on theoretical grounds (57). Although charge fluctuation forces must certainly occur, they appear to be negligible at least for liquid crystal formation in monovalent-ion solutions. At these larger separations, the double-layer repulsion often couples with configurational fluctuations to create exponentially decaying forces whose decay length is significantly larger than the expected Debye screening length (42).

Bare short-range molecular interactions between DNA molecules appear to be insensitive to the amount of added salt. This has been taken as evidence that they are not electrostatic in origin, as attested also by similar interactions between completely uncharged polymers such as schizophilline (Fig. 5). The term hydration force associates these forces with perturbations of the water structure around DNA surface (54). Alternatively, short-range repulsion has been viewed as a consequence of the electrostatic force specific to high DNA density and counterion concentration (58).

F. High-density DNA Mesophases

Ordering of DNA can be induced by two alternative mechanisms. First of all, attractive interactions between different DNA segments can be enhanced by adding multivalent counterions believed to promote either counterion correlation forces (59) or electrostatic (60) and hydration attraction (22). In these cases, DNA aggregates spontaneously. Alternatively, one can add neutral crowding polymers to the bathing solution that phase separate from DNA and exert osmotic stress on the DNA subphase (61). In this case the intersegment repulsions in DNA are simply counteracted by the large externally applied osmotic pressure. DNA is forced in this case to condense under externally imposed constraints. This latter case is formally (but only formally) analogous to a Boyle gas pressure experiment but with osmotic pressure playing the role of ordinary pressure. The main difference being that ordinary pressure is set mechanically, whereas osmotic pressure has to be set through the chemical potential of water, which is in turn controlled by the amount of neutral crowding polymers (e.g., PEG, PVP, dextran) in the bathing solution (55).

At very high DNA densities, where the osmotic pressure exceeds 160 atm, DNA can exist only in a (poly)crystalline state (62). Nearest neighbors in such an array are all oriented in parallel and show correlated (nucleotide) base stacking between neighboring duplexes (Figs. 11 and 14). This means that there is a long-range correlation in the positions of the backbone phosphates between different DNA molecules in the crystal. The local symmetry of the lattice is monoclinic. Because of the high osmotic pressure, DNA is actually forced to be in an A conformation characterized by a somewhat larger outer diameter as well as a somewhat smaller pitch than in the canonical B conformation (see Fig. 12), which persists at smaller densities. If the osmotic pressure of such a crystal is increased above 400 atm, the helix begins to crack and the sample loses structural homogeneity (62).

Lowering the osmotic pressure does not have a pronounced effect on the DNA crystal until it is down to ~160 atm. Then the crystal as a whole simultaneously expands while individual DNA molecules undergo an A-B conformational transition (see Fig. 14) (62). This phase transformation is thus first order, and besides being a conformational transition for single DNAs, is connected also with the melting of the base stacking as well as positional order of the helices in the lattice. The ensuing low-density mesophase, where DNA is in the B conformation, is therefore characterized by short range base stacking order, short range 2-dimensional positional order and long-range bond orientational order (Fig. 15) (63). This order is connected with the spatial direction of the nearest neighbors (64). It is for this reason that the phase has been termed a ''line hexatic'' phase. Hexatics usually occur only in 2-dimensional systems. They have crystalline bond orientational order but liquidlike positional order. There might be a hexatic-hexagonal columnar transition somewhere along the hexatic line, though a direct experimental proof is lacking.

The difference between the 2 phases is that the hexagonal columnar phase has also a crystalline positional order and is thus a real 2-dimensional crystal (see Fig. 15) (65). It is the long-range bond orientational order that gives the line hexatic phase some crystalline character (66). The DNA duplexes are still packed in parallel, while the local symmetry perpendicular to the long axes of the molecules is changed to hexagonal. The directions of the nearest neighbors persist through macroscopic dimensions (on the order of mm) while their positions tend to become disordered already after several (typically 5 to 10) lattice spacings. This mesophase has a characteristic X-ray scattering fingerprint (see Fig. 15). If the X-ray beam is directed parallel to the long axis of the molecules, it will show a hexagonally symmetric diffraction pattern of broad liquidlike peaks (67).

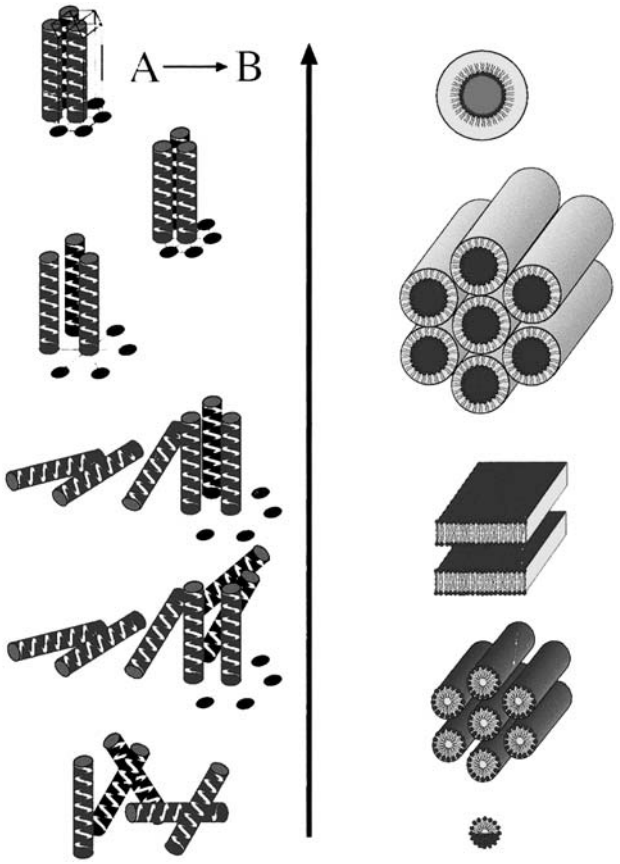


Figure 14 Schematic phase diagrams for DNA (left) and lipids (right). In both cases, the arrow indicates increasing density in both cases. DNA starts (bottom) as a completely disordered solution. It progresses through a sequence of blue phases characterized by cholesteric pitch in two perpendicular directions (68), then to a cholesteric phase with pitch in only one direction. At still larger densities, this second cholesteric phase is succeeded by a hexatic phase, characterized by short-range, liquidlike positional order and long-range, crystallike bond orientational (or hexatic order, indicated by lines). At highest densities, there is a crystalline phase characterized by long-range positional order of the molecules and long-range base stacking order in the direction of the long axes of the molecules. Between the hexatic and the crystalline forms, there might exist a hexagonal columnar liquid-crystalline phase, that is similar to a crystal, but with base stacking order only on short scales. The lipid-phase diagram (77) is a composite of results obtained for different lipids. It starts from a micellar solution and progresses through a phase of lipid tubes to a multilamellar phase of lipid bilayers. This is followed by an inverted hexagonal columnar phase of water cylinders and possibly goes to an inverted micellar phase. Most lipids show only a subset of these possibilities. Boundaries between the phases shown here might contain exotic cubic phases not included in this picture. See the color insert for a color version of this figure.

Typical lattice spacings in the line hexatic phase are between 25 and 35 Å (i.e., between 600 and 300 mg/mL of DNA) (63). The free energy in this mesophase is mostly a consequence of the large hydration forces stemming from removal of water from the phosphates of the DNA backbone. Typically independent of the ionic strength of the bathing solution, these hydration forces (54) depend exponentially on the interhelical separation and decay with a decay length of about 3 Å (11) at these large densities. This value of the hydration decay length seems to indicate that it is determined solely by the bulk properties of the solvent (i.e., water).

It is interesting to note that the behavior of short-fragment DNA in this range of concentrations is different from the long DNA (65). The short-fragment DNA, typically the nucleosomal DNA fragment of 146 bp, makes a 2-dimesional hexagonal phase at interaxial spacing of ~30 Å, that progressively orders into a 3-dimensional hexagonal phase on decrease of the interaxial spacing to ~23 Å (65). At still larger concentrations, the short-fragment DNA makes a 3-dimensional orthorhombic crystal, with a deformed hexagonal unit cell perpendicular to the *c*-axis. Concurrently to this symmetry transformation, the helical pitch of the condensed phase decreases continuously from 34.6 to 30.2 Å (65). The reasons for this fundamental difference between the behavior of long as opposed to short-fragment DNA is still not well understood.

When the osmotic pressure is lowered to about 10 atm (corresponding to interaxial spacing of about 35 Å, or DNA density of about 300 mg/mL), the characteristic hexagonal X-ray diffraction fingerprint of the line hexatic mesophase disappears continuously. This disappearance suggests the presence of a continuous, second-order transition into a low-density cholesteric (63). It is characterized by short-range (or effectively no) base stacking order, short-range positional order, short-range bond orientational order, but long-range cholesteric order, manifested in a continuing rotation of the long axis of the molecules in a preferred direction. In this sense, the cholesteric DNA mesophase would retain the symmetry of a 1-dimensional crystal. X-ray diffraction pattern of the DNA in the cholesteric phase is isotropic and has the form of a ring. Crossed polarizers, however, reveal the existence of long-range cholesteric order just as in the case of short chiral molecules. The texture of small drops of DNA cholesteric phase (spherulites) under crossed polarizers (Fig. 16) reveals the intricacies of orientational packing of DNA, where its local orientation is set by a compromise between interaction forces and macroscopic geometry of a spherulite. It is thus only at these low densities that the chiral character of the DNA finally makes an impact on the symmetry of the mesophase. It is not yet fully understood why the chiral order is effectively screened from the high-density DNA mesophases.

At still smaller DNA densities, the predominance of the chiral interactions in the behavior of the system remains. Recent work on the behavior of low-density DNA mesophases indicates (68) that the cholesteric part of the phase diagram might end with a sequence of blue phases that would emerge as a consequence of the loosened packing constraints coupled to the chiral character of the DNA molecule. At DNA density

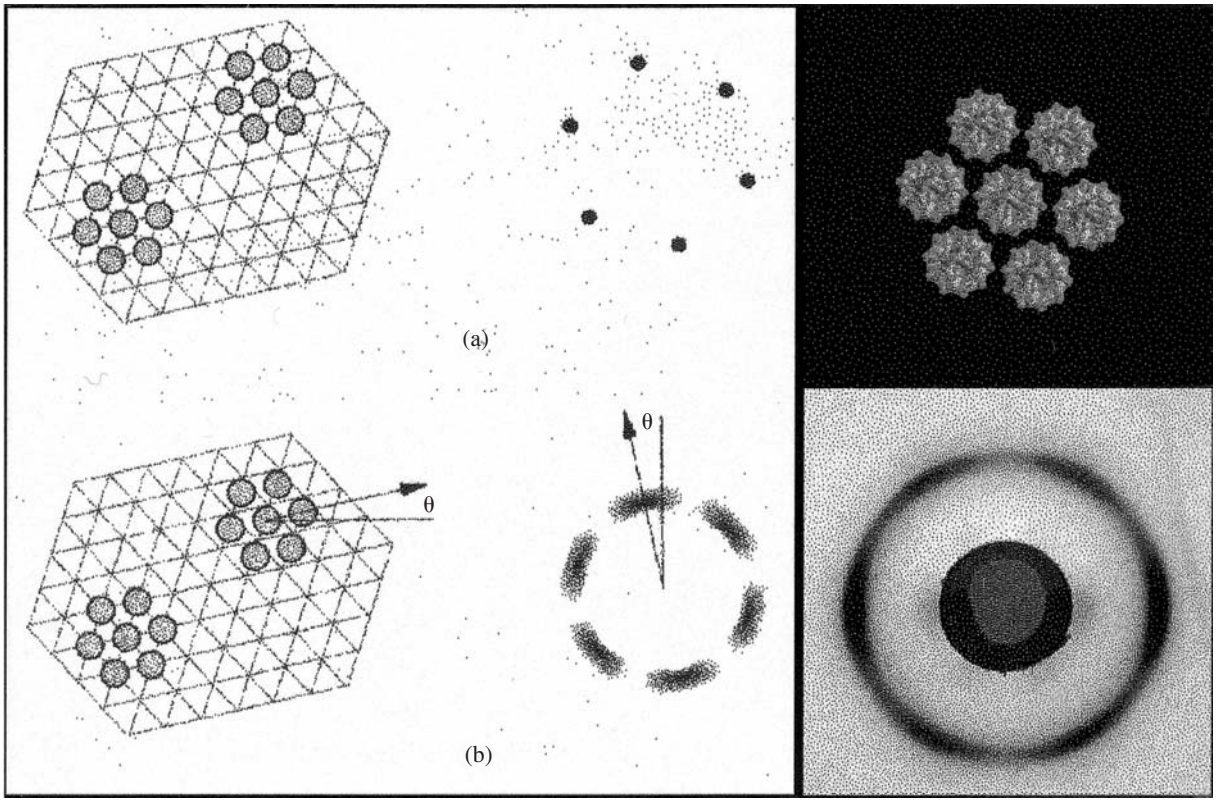


Figure 15 Bond orientational or hexatic order. With a real crystal if one translates part of the crystal by a lattice vector, the new position of the atoms completely coincides with those already there. (Adapted from ref. 67.) In a hexatic phase the directions to the nearest neighbors (bond orientations) coincide (after rotation by 60°), but the positions of the atoms do not coincide after displacement in 1 of the 6 directions! Consequently, a real crystal gives a series of very sharp Bragg peaks in X-ray scattering (upper half of box), whereas a hexatic gives hexagonally positioned broad spots. The pattern of X-ray scattering by high- density DNA samples gives a fingerprint of a hexatic phase. The densitogram of the scattering intensity (right half of figure) shows 6 pronounced peaks that can be Fourier decomposed with a marked sixth-order Fourier coefficient, another sign that that the scattering is due to long-range bond orientational order (63). See the color insert for a color version of this figure.

of about 10 mg/mL, the cholesteric phase line would end with DNA reentering the isotropic liquid solution where it remains at all subsequent densities, except perhaps at very small ionic strengths (69).

G. DNA Equation of State

The free energy of the DNA cholesteric mesophase appears to be dominated by the large elastic shape fluctuations of its constituent DNA molecules (70) that leave their imprint in the very broad X-ray diffraction peak (55). Instead of showing the expected exponential decay characteristic of screened electrostatic interactions (71), where the decay length is equal to the Debye length, it shows a fluctuation-enhanced repulsion similar to the Helfrich force existing in the flexible smectic multilamellar arrays (41). Fluctuations not only boost the magnitude of the existing screened electrostatic repulsion, but also extend its range through a modified decay length equal to 4 times the Debye length. The factor-of-4 enhancement in the range of the repulsive force is a consequence of the coupling between the bare electrostatic repulsions of exponential type and the thermally driven elastic shape fluctuations described through elastic curvature energy that is proportional to the square of the second derivative of the local helix position (42). In the last instance, it is a consequence of the fact that DNAs in the array interact via an extended, soft-screened electrostatic potential and not through hard bumps as assumed in the simple derivation in Chapter 2.

The similarity of the free energy behavior of the smectic arrays with repulsive interactions of Helfrich type and the DNA arrays in the cholesteric phase that can also be understood in the framework of the Helfrich-type-enhanced repulsion satisfies a consistency test for our understanding of flexible supermolecular arrays.

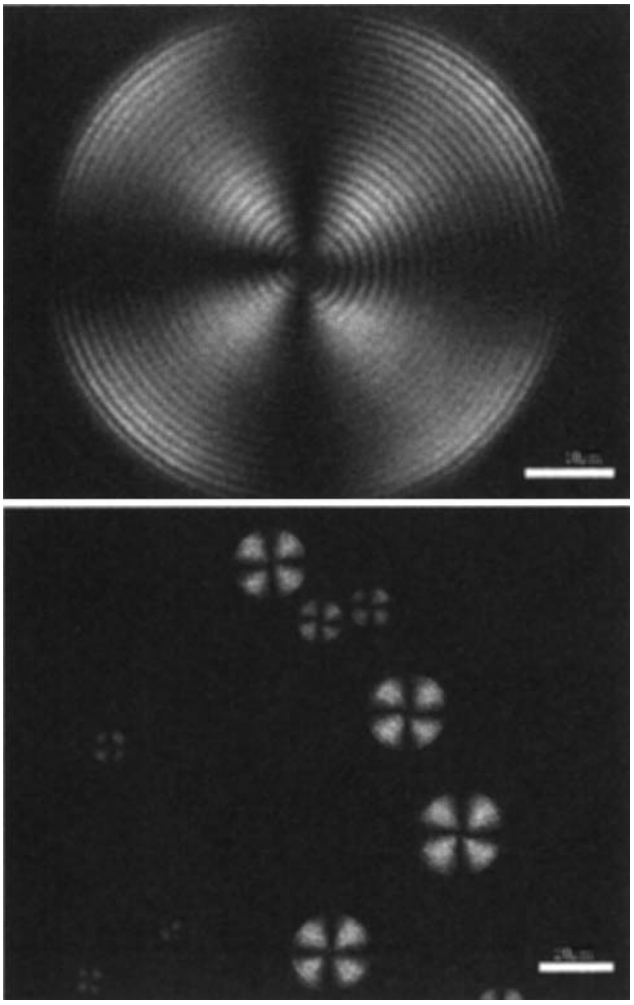


Figure 16 Texture of small drops of DNA cholesteric phase (spherulites) in a PEG solution under crossed polarizers. These patterns reveal the intricacies of DNA orientational packing when its local orientation is set by a compromise between interaction forces and the macroscopic geometry of a spherulite. The change from a bright to a dark stripe indicates that the orientation of the DNA molecule has changed by 90 degrees.

IV. LIPID MESOPHASES

A. Aggregation of Lipids in Aqueous Solutions

Single-molecule solutions of biological lipids exist only over a negligible range of concentrations; virtually all interesting lipid properties are those of aggregate mesophases such as bilayers and micelles. Lipid molecules cluster into ordered structures to maximize hydrophilic and minimize hydrophobic interactions (72,73). These interactions include negative free energy contribution from the solvation of polar heads and van der Waals interactions of hydrocarbon chains, competing with positive contributions such as steric, hydration, and electrostatic repulsions between polar heads. The ''hydrophobic effect,'' which causes segregation of polar and nonpolar groups, is said to be driven by the increase of the entropy of the surrounding medium.

Intrinsic to the identity of surfactant lipids is the tension between water-soluble polar groups and lipid-soluble hydrocarbon chains. There is no surprise then that the amount of water available to an amphiphile is a parameter pertinent to its modes of packing and to its ability to incorporate foreign bodies.

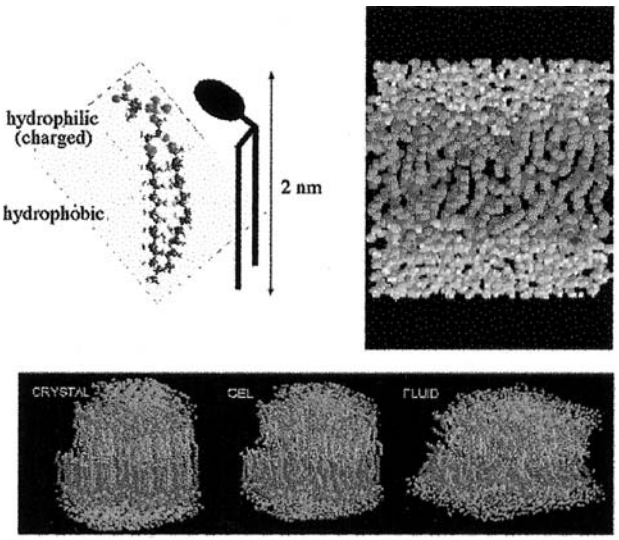
These interactions therefore force lipid molecules to self-assemble into different ordered microscopic structures, such as bilayers, micelles (spherical, ellipsoidal, rodlike, or disk-like), which can, especially at higher concentrations, pack into macroscopically ordered phases, such as lamellar, hexagonal, inverted hexagonal, and cubic. The morphology of these macroscopic phases changes with the balance between attractive van der Waals and ion correlation forces vs. electrostatic, steric, hydration, and undulation repulsion (74).

B. The Lipid Bilayer

The workhorse of all lipid aggregates is the bilayer (Fig. 17) (73). This sandwich of two monolayers, with nonpolar hydrocarbon chains tucked in toward each other and polar groups facing water solution, is only about 20 to 30 Å thick. Yet it has the physical resilience and the electrical resistance to form the plasma membrane that divides ''in'' from ''out'' in all biological cells. Its mechanical properties have been measured in terms of bending and stretching moduli. These strengths together with measured interactions between bilayers in multilamellar stacks have taught us to think quantitatively about the ways in which bilayers are formed and maintain their remarkable stability.

With some lipids, such as double-chain phospholipids, when there is the need to encompass hydrocarbon components voluminous compared with the size of polar groups, the small surface-to-volume ratio of spheres, ellipsoids, or even cylinders cannot suffice even at extreme dilution. Bilayers in this case are the aggregate form of choice. These may occur as single ''unilamellar'' vesicles, as onionlike multilayer vesicles, or multilamellar phases of indefinite extent. In vivo, bilayer-forming phospholipids create the flexible but tightly sealed plasma-membrane matrix that defines the inside from the outside of a cell. In vitro, multilayers are often chosen as a matrix of choice for the incorporation of polymers. Specifically, there are tight associations between positively charged lipids that merge with negatively charged DNA in a variety of forms (see below).

The organization of lipid molecules in the bilayer itself can vary (73). At low enough temperatures or dry enough conditions the lipid tails are frozen in an all-trans conformation that minimizes the energy of molecular bonds in the alkyl tails of the lipids. Also, the positions of the lipid heads along the surface of the bilayer are frozen in 2-dimensional positional order, making the overall conformation of the lipids in



Lipid	Bending rigidity [10^{-19}J]	Area compression [mN/m]
DMPC	1.15	145
SOPC	0.9	200
EggPC	0.4-2	
Plasma membrane	0.2-2	700
Red Blood Cell	0.13 - 0.3	450

Figure 17 The lipid bilayer. A lipid molecule has a hydrophilic and a hydrophobic part (here shown is the phosphatidylserine molecule that has a charged headgroup). At high-enough densities, lipid molecules assemble into a lipid bilayer. Together with membrane proteins as its most important component the lipid bilayer is the underlying structural component of biological membranes. The degree of order of the lipids in a bilayer depends drastically on temperature and goes through a sequence of phases (see main text): crystalline, gel, and fluid, depicted in the middle drawing. The box at bottom gives sample values of bilayer bending rigidity and area compressibility for some biologically relevant lipids and one well-studied cell membrane. See the color insert for a color version of this figure.

the bilayer crystal (L_C). The chains can either be oriented perpendicular to the bilayer surface (L_β and $L_{\beta'}$) or be tilted (crystalline phase L_C or ripple phase P_β). Such a crystalline bilayer cannot exist by itself but assembles with others to make a real 3-dimensional crystal.

Upon heating, various rearrangements in the 2-dimensional crystalline bilayers occur, first the positional order of the headgroups melts leading to a loss of 2-dimensional order ($L_{\beta'}$) and tilt (L_β), then, at the gel–liquid crystal phase transition the untilted or rippled (P_β phase) bilayer changes into a bilayer membrane with disordered polar heads in 2 dimensions and conformationaly frozen hydrocarbon chains, allowing them to spin around the long axes of the molecules, the so-called L_α phase. At still higher temperatures, the thermal disorder finally also destroys the ordered configuration of the alkyl chains, leading to a fluidlike bilayer phase. The fluid bilayer phase creates the fundamental matrix that according to the fluid mosaic model (72) contains different other ingredients of biological membranes (e.g., membrane proteins, channels, etc.).

Not only bilayers in multilamellar arrays but also liposome bilayers can undergo such phase transitions; electron microscopy has revealed fluid phase, rippled, and crystalline phase in which spherical liposomes transform into polyhedra due to very high values of bending elasticity of crystallized bilayers (75).

The fluid phase of the lipid bilayer is highly flexible. This flexibility makes it prone to pronounced thermal fluctuations, resulting in large excursions away from a planar shape. This flexibility of the bilayer is essential for understanding the zoo of equilibrium shapes that can arise in closed bilayer (vesicles) systems (76). Also, just as in the case of flexible DNA, it eventually leads to configurational entropic interactions between bilayers that have been crammed together (41). Bilayers and linear polyelectrolytes thus share a substantial amount of fundamentally similar physics that allows us to analyze their behavior in the same framework.

C. Lipid Polymorphism

Low temperature phases (77) are normally lamellar with frozen hydrocarbon chains tilted (crystalline phase L_C or ripple phase P_β) or nontilted (L_β and $L_{\beta'}$ form three-, two-, or one-D crystalline or gel phases) with respect to the plane of the lipid bilayers. Terminology from thermotropic liquid crystals phenomenology (50) can be used efficiently in this context: these phases are smectic, and SmA describes 2-dimensional fluid with no tilt while a variety of SmC phases with various indices encompass tilted phases with various degrees of 2-dimensional order. Upon melting, liquid crystalline phases with 1- (lamellar L_α), 2- (hexagonal II), or 3-dimensional (cubic) positional order can form.

The most frequently formed phases are micellar, lamellar, and hexagonal (Fig. 14). Normal hexagonal phase consists of long cylindrical micelles ordered in a hexagonal array, while in the inverse hexagonal II (H_{II}) phase water channels of inverse micelles are packed hexagonally with lipid tails filling the interstices. In excess water, such arrays are coated by a lipid monolayer. The morphology of these phases can be maintained upon their (mechanical) dispersal into colloidal dispersions. Despite that energy has to be used to generate dispersed mesophases relatively stable colloidal dispersions of particles with lamellar, hexagonal, or cubic symmetry can be formed.

Many phospholipids found in lamellar cell membranes, after extraction, purification, and resuspension, prefer an inverted hexagonal geometry (Fig. 18) (77). Under excess-water conditions different lipids will assume different most-favored spontaneous radii for the water cylinder of this inverted phase (78). An immediate implication is that different lipids are strained to different degrees when forced into lamellar pack-

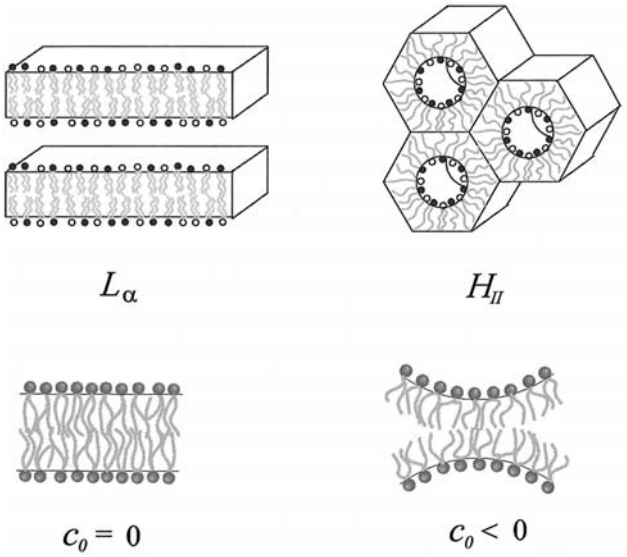


Figure 18 Different lipids are strained to different degrees when forced into lamellar packing. Relaxation of this strain contributes to the conditions for lamellar–to–inverted hexagonal phase transitions that depend on temperature, hydration, and salt concentration (for charged lipids). See the color insert for a color version of this figure.

ing. There are lamellar-inverted hexagonal phase transitions that occur with varied temperature, hydration, and salt concentration (for charged lipids) that form in order to alleviate this strain (see Fig. 18).

In the presence of an immiscible organic phase emulsion, droplets can assemble (79). In regions of phase diagram that are rich in water, oil-in-water emulsions and microemulsions ($c > 0$) can be formed, while in oil-rich regions these spherical particles have negative curvature and are therefore water-in-oil emulsions. The intermediate phase between the two is a bicontinuous emulsion that has zero average curvature and an anomalously low value of the surface tension (usually brought about the use of different cosurfactants) between the two immiscible components. Only microemulsions can form spontaneously (analogously to micelle formation) while for the formation of a homogeneous emulsion some energy has to be dissipated into the system.

The detailed structure of these phases as well as the size and shape of colloidal particles are probably dominated by:

- The average molecular geometry of lipid molecules
- Their aqueous solubility and effective charge
- Weaker interactions such as intra- and intermolecular hydrogen bonds
- Stereoisomerism as well as interactions within the medium

All depend on the temperature, lipid concentration, and electrostatic and van der Waals interactions with the solvent and solutes. With charged lipids, counterions, especially anions, may also be important. Ionotropic transitions have been observed with negatively charged phospholipids in the presence of metal ions leading to aggregation and fusion (80). In cationic amphiphiles, it was shown that simple exchange of counterions can induce micelle-vesicle transition. Lipid polymorphism is very rich and even single-component lipid systems can form a variety of other phases, including ribbonlike phases, coexisting regions and various stacks of micelles of different shapes.

D. Forces in Multilamellar Bilayer Arrays

Except for differences in dimensionality, forces between bilayers are remarkably similar to those between DNA. At very great separations between lamellae, the sheetlike structures flex and ''crumple'' because of (thermal) Brownian motion (41). Just as an isolated flexible linear polymer can escape from its 1 linear dimension into the 3 dimensions of the volume in which it is bathed, so can 2-dimensional flexible sheets. In the most dilute solution, biological phospholipids will typically form huge floppy closed vesicles; these vesicles enjoy flexibility while satisfying the need to keep all greasy nonpolar chains comfortably covered by polar groups rather than exposed at open edges. For this reason, in very dilute solution, the interactions between phospholipid bilayers are usually space wars of collision and volume occupation. This steric competition is always seen for neutral lipids; it is not always true for charged lipids (74).

Especially in the absence of any added salt, planar surfaces emit far-ranging electrostatic fields (27) that couple to thermally excited elastic excursions to create very long-range repulsion (44,83). As with DNA, this repulsion is a mixture of direct electrostatic forces and soft collisions mediated by electrostatic forces rather than by actual bilayer contact. In some cases electrostatic repulsion is strong enough to snuff out bilayer bending when bilayers form ordered arrays with periodicities as high as hundreds of Å (82).

Almost always bilayers align into well-formed stacks when their concentration approaches ~50 to 60 weight percent and their separation is brought down to a few tens of Å. In this region charged layers are quite orderly with little lamellar undulation. In fact, bilayers of many neutral phospholipids often spontaneously fall out of dilute suspension to form arrays with bilayer separations between 20 and 30 Å. These spontaneous spacings are believed to reflect a balance between van der Waals attraction and undulation-enhanced hydration repulsion (74). One way to test for the presence of van der Waals forces has been to add solutes such as ethylene glycol, glucose, or sucrose to the bathing solutions. It is possible then to correlate the changes in spacing with changes in van der Waals forces due to the changes in dielectric susceptibility as described above (83). More convincing, there have been direct measurements of the work to pull apart bilayers that sit at spontaneously assumed spacings. This work of separation is of the magnitude expected for van der Waals attraction. (84).

Similar to DNA, multilayers of charged or neutral lipids subjected to strong osmotic stress reveal exponential variation in osmotic pressure vs. bilayer separation (74). Typically at separations between dry ''contact'' and 20 Å, exponential decay lengths are 2 to 3 Å in distilled water or in salt solution, whether phospholipids are charged or neutral. Lipid bilayer repulsion in this range is believed to be due to the work of polar group dehydration sometimes enhanced by lamellar collisions from thermal agitation (85). Normalized per area of interacting surface the strength of hydration force acting in lamellar lipid arrays and DNA arrays is directly comparable.

Given excess water, neutral lipids will usually find the above-mentioned separation of 20 to 30 Å at which this hydration repulsion is balanced by van der Waals attraction. Charged lipids, unless placed in solutions of high salt concentration, will swell to take up indefinitely high amounts of water. Stiff charged bilayers will repel with exponentially varying electrostatic double layer interactions, but most charged bilayers will undulate at separations where direct electrostatic repulsion has weakened. In that case, similar to what has been described for DNA, electrostatic repulsion is enhanced by thermal undulations (86).

E. Equation of State of Lipid Mesophases

Lipid polymorphism shows much less universality that DNA. This is of course expected because lipid molecules come in many different varieties (73) with strong idiosyncrasies in terms of the detailed nature of their phase diagrams. One thus can not achieve the same degree of generality and universality in the description of lipid phase diagram and consequent equations of state as was the case for DNA.

Nevertheless, recent extremely careful and detailed work on PCs by J. Nagle and his group (87) points strongly to the conclusion that at least in the lamellar part of the phase diagram of neutral lipids the main features of the DNA and lipid membrane assembly physics indeed is the same (85). This statement however demands qualification. The physics is the same, provided one first disregards the dimensionality of the aggregates—1 dimensional in the case of DNA and 2 dimensional in the case of lipid membranes—and takes into account the fact that while van der Waals forces in DNA arrays are negligible, they are essential in lipid membrane force equilibria. One of the reasons for this state of affairs is the large difference, unlike in the case of DNA, between the static dielectric constant of hydrophobic bilayer interior, composed of alkyl lipid tails, and the aqueous solution bathing the aggregate.

We have already pointed out that in the case of DNA arrays quantitative agreement between theory, based on hydration and electrostatic forces augmented by thermal undulation forces, and experiment has been obtained and extensively tested (7,42). The work on neutral lipids (85) claims that the same level of quantitative accuracy can be achieved also in lipid membrane assemblies if one takes into account hydration and van der Waals forces again augmented by thermal undulations. Of course, the nature of the fluctuations in the 2 systems is different and is set by the dimensionality of the fluctuating aggregates—1- vs. 2-dimensional.

The case of lipids adds an additional twist to the quantitative link between theory and experiments. DNA in the line hexatic as well as cholesteric phases (where reliable data for the equation of state exist) is essentially fluid as far as positional order is concerned and thus has unbounded positional fluctuations. Lipid membranes in the smectic multilamellar phase are quite different in this respect. They are not really fluid as far as positional order is concerned but show something called quasilong range (QLR) order, meaning that they are in certain respects somewhere between a crystal and a fluid (50,67). The quasi long-range positional order makes itself recognizable through the shape of the X-ray diffraction peaks in the form of persistent (Caille) tails (67).

In a crystal one would ideally expect infinitely sharp peaks with Gaussian broadening only because of finite accuracy of the experimental setup. Lipid multilamellar phases, however, show peaks with very broad, non-Gaussian, and extended tails that are one of the consequences of QLR positional order. The thickness of these peaks for different orders of X-ray reflexions varies in a characteristic way with the order of the reflexion (67). It is this property that allows us to measure not only the average spacing between the molecules, but also the amount of fluctuation around this average spacing. Luckily, the theory also predicts that and without any free parameters (all of them being already determined from the equation of state) the comparison between predicted and measured magnitude in positional fluctuations of membranes in a multilamellar assembly is more than satisfactory (85).

In summing up, the level of understanding of the equation of state reached for DNA and neutral lipid membrane arrays is pleasing.

V. DNA–LIPID INTERACTIONS

Mixed in solution with cationic lipids (CLs), DNA spontaneously forms CL-DNA aggregates of submicron size. These DNA-lipid aggregates, sometimes called ''lipoplexes,'' (88) are routinely used for cell transfection in vitro. More important, they are used primarily as potential gene delivery vehicles for in vivo gene therapy [for recent reviews, see (89–94) and references therein]. Under appropriate conditions these aggregates reveal complex underlying thermodynamic phase behavior. There is a practical paradox here. We use stable equilibrium structures to reveal the forces that cause aggregation and assembly; we use this knowledge of forces to create the unstable preparations likely to be most efficient in transfection.

Lipoplexes for transfection were first proposed by Felgner and coworkers (95,96). The guiding idea was to overcome the electrostatic repulsion between cell membranes (containing negatively charged lipids) and negative DNA by complexing DNA with positively charged CL. Preliminary experimental data showed that at least some lipoplexes deliver DNA through direct fusion with the cell membrane (97). More often,

however, lipoplex internalization probably proceeds through endocytosis after initial interaction with the cell's membrane.

Prior to the attempts to use lipoplexes for transfection, studies of DNA aggregated with multivalent cations and coated with negatively charged liposomes were also explored as possible vectors. It was hoped that CL-DNA complexes would no longer require an additional complexing agent, and that also, the transfection efficiency would be higher. The complex's lipid coating could protect the tightly packed DNA cargo during its passage to the target cells.

Although not confronted with the immunological response, risked by the alternative viral vector strategy, the use of lipoplexes in gene therapy is still hampered by toxicity of the CL and low in vivo tranfection efficiency, despite the in vitro efficiency of some CL formulations. This discrepancy can be attributed to the multistage and multibarrier process the complexes must endure before transfection is achieved. These steps typically include passage in the serum, interaction with target and other cells, internalization, complex disintegration in the cytoplasm, transport of DNA into the nucleus, and ultimately expression.

In the search for increasingly more potent gene delivery vectors, the intimate relationship between the lipoplex's phase structure (or morphology) and its transfection efficiency probably serves as the greatest motivation for their study. How is transfection affected by lipoplex morphology? How may this structure be controlled? Experiment and theory of the past decade shed some light on such fundamental questions. They may give perspective for future strategies to design CL-based nonviral vectors.

To this end, we present our current understanding of the structure and phase behavior of CL-DNA complexes. We review the relation of structure to transfection efficiency and, more specifically, to the way the complex formation overcomes one barrier to DNA release into the cytoplasm.

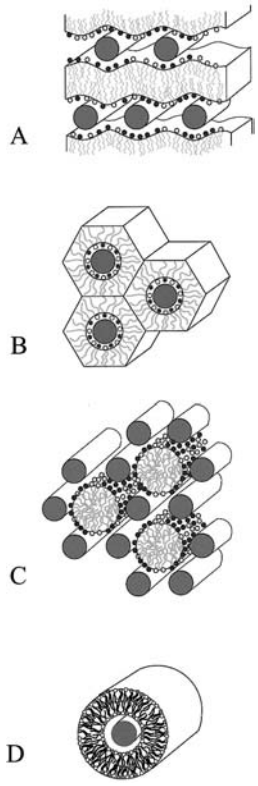


Figure 19 Schematic illustration of some possible structures of DNA–mixed lipid (cationic/nonionic) complexes. (A) The sandwichlike (L_α^c) lamellar complex composed of parallel DNA molecules intercalated between lipid bilayers. (B) The honeycomblike (H_{II}^c) hexagonal complex, composed of a hexagonally packed bundle of monolayer-coated DNA strands. (C) Two interpenetrating hexagonal lattices, one of DNA, the other of micelles. (D) Spaghetti-like complex, composed of bilayer-coated DNA. (Reprinted by permission from Ref. 143, Biophysical Society.) See the color insert for a color version of this figure.

A. Structure of CL-DNA Complexes

In general, the structures of CL-DNA composite phases can be viewed as morphological hybrids of familiar pure-lipid and pure-DNA phases. A first example is the lamellarlike structure initially proposed by Lasic at al. (99,100). The first comprehensive and unambiguous evidence for this structure came from a series of studies by Rädler et al. (101–105). From high-resolution synchrotron X-ray diffraction and optical microscopy, they reported the existence of novel lamellar CL-DNA phase morphologies. In particular, one complex structure was shown to consist of lamellar multilayer. In this case smecticlike stacks of mixed bilayers, each composed of a mixture of CL—for example, dioleoyltrimethylammonium propane (DOTAP)—and neutral ''helper'' lipid—for example, dioleoylphosphatidylcholin (DOPC)—with monolayers of DNA strands intercalated within the intervening water gaps (Fig. 19A), like a multilipid bilayer L_α phase (106). Helper lipids are often added for their fusogenic properties. Dioleoylphosphatidylethanol amine (DOPE), for example, is conjectured to promote transfection. In addition, because pure (synthetically derived) cationic lipids often tend to form micelles in solution, helper lipids facilitate the formation of membranes.

In this L_α^c complex geometry, the DNA strands within each gallery are parallel to each other, exhibiting a well-defined repeat distance d. While d depends on the CL/DNA and CL/HL concentration ratios, the spacing between two apposed lipid monolayers is nearly constant at ~26 Å, corresponding to the diameter of double-stranded B-DNA, ca. 20 Å, surrounded by a thin hydration shell. This L_α^c lamellar (''sandwich'') complex is stabilized by the electrostatic attraction between the negatively charged DNA and the cationic lipid bilayer. Because of strong electrostatic repulsion between the charged bilayers (particularly at low salt conditions), the lamellar lipid phase is unstable without DNA.

Quite different equilibrium ordered phase morphologies were found to occur from other choices of neutral helper lipid (HL). In the case of DOPE, or lecithin, for example, inverted hexagonal (''honeycomb'' or H_{II}^{c}) organization of the lipid, with stretches of double-stranded DNA lying in the aqueous solution regions, were found to form, see Fig. 19B (95,102,107). The H_{II}^{c} structure may be regarded as the inverse-hexagonal (H_{II}) lipid phase, with DNA strands wrapped within its water tubes. Here, too, the diameter of the water tubes is only slightly larger than the diameter of the DNA ''rods.'' The presence of DNA is crucial for stabilizing the hexagonal structure. Without it, strong electrostatic repulsion will generally drive the lipids to organize themselves into planar bilayers. In fact, the most abundant aggregate structure of pure CL and HL mixtures, from which hexagonal complexes are subsequently formed, is single-bilayer liposomes.

Other CL-DNA phases have also been observed. One of the earliest studies probing the structure of lipoplexes showed some evidence for an hexagonal arrangement of rodlike micelles intercalated between hexagonally packed DNA, Fig. 19C (108,109). The number of possibilities is even larger if one also considers metastable intermediates. The ''spaghetti'' structure (see Fig. 19D), observed using freeze-fracture electron microscopy, has been predicted by theory to probably be one such metastable morphology (110,111). Here, each (possibly supercoiled) DNA strand is coated by a cylindrical bilayer of the CL/HL lipid mixture (112,113). Early proposed models of the CL-DNA complexes suggested a ''beads on a string'' type complex, in which the DNA is wrapped around or in between lipid vesicles (and even spherical micelles). Although this may not turn out to be an equilibrium structure, such aggregates are sometimes found, and may also serve as unstable intermediates (114–116). Other structures, such as the bilamellar invaginated liposomes (BIV) made of DOTAP-Chol, have been proposed and demonstrated to be efficient vectors (97,117). These structures resemble to some degree the L_{α}^{c} phase. However, formed from extruded liposomes, the BIVs are most probably metastable.

What factors determine which of these phases (or possibly several coexisting structures) actually form in solution? To what degree can we control and predict them? Control can first be achieved through the choice of type of CL and HL, and the ratio between the 2 used in forming liposomes. This in turn will determine such basic properties as the lipid bilayer's bending rigidity, spontaneous curvature, and surface charge density of the water–lipid aggregate interface. An additional experimentally controllable parameter is the ratio between the lipid and DNA content in solution. Both these parameters, we show, have significant effects on the phases that are formed.

B. Counterion Release

From the start, it was realized that the expected condensation of DNA with oppositely charged lipids could be used to package and send DNA to transfect targeted cells. The expectation that the DNA and lipids would aggregate was intuitively based on the notion that oppositely charged bodies attract. Early experiments confirmed the aggregation of DNA and lipids. However, the mechanism by which CL and DNA were found to associate—previously termed in the context of macromolecular association ''counterion release'' (118)—is more intricate than the ''opposites attract'' mechanism that may be naively expected.

Prior to association, DNA and lipids are bathed in the aqueous solutions containing their respective counterions, so that the solutions are overall electrostatically neutral. The counterions are attracted to the oppositely charged macromolecules, thus gaining electrostatic energy. Here, in addition to DNA, we also refer to the preformed CL liposomes as a ''macromolecules'' because they typically retain their integrity in solution, even upon association with other charged macromolecules. The counterions are therefore confined to the vicinity of the oppositely charged macromolecules at the compromise of greater translational entropy in solution.

Upon association, the 2 oppositely charged macromolecules condense to form CL-DNA complexes (Fig. 20). Many (possibly all) previously confined counterions can now be expelled into the bulk solution from the lipoplex interior, thus gaining translational entropy. Although the translational entropy of the paired macromolecules is reduced by (typically) only a few k_BTs (due to loss of conformational and translational entropy), many released counterions can now favorably contribute to a gain in entropy, each by a comparable amount. For this reason it is sometimes stated that the DNA–lipid condensation is ''entropically driven.'' The electrostatic en-

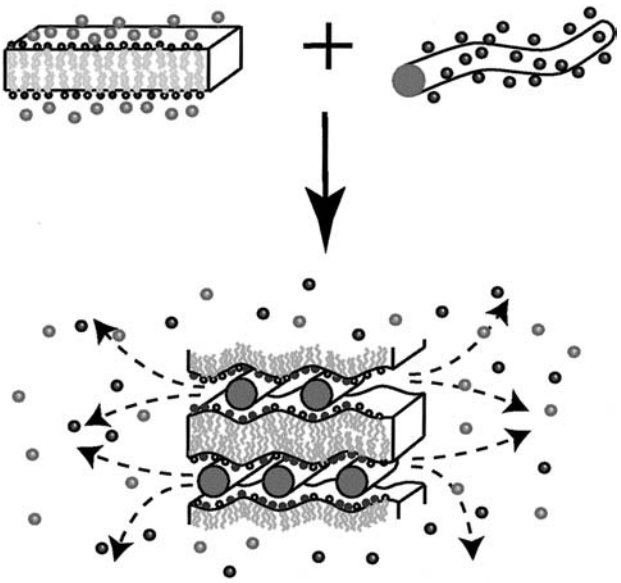


Figure 20 Schematic illustration of the condensation of DNA and lipid bilayers (liposomes) into CL-DNA complexes. In the process, the previously confined counterions are released into the bathing solution, thereby gaining translational entropy. See the color insert for a color version of this figure.

ergy can also contribute somewhat to stabilizing the lipoplexes. However, it has been well argued, both experimentally and theoretically, that the cardinal contribution to the association free energy of CL–DNA complexes is the entropy gain associated with counterion release (119,120).

Further support was given by counting released ions, using conductivity measurements of the supernatant. It was possible to determine that a maximal number of counterions were released when the number of ''fixed'' charges on the DNA and lipid were exactly equal.

Calorimetric measurements confirm this finding and find furthermore that the association could in fact be endothermic, so that it is only favorable for entropic reasons (121,122). The special point at which the number of positive and negative fixed charges is equal has been termed the ''isoelectric point.'' At this point, the (charging) free energy of the complex is minimal: the fixed charges of opposite signs fully compensate each other, thus allowing essentially all the counterions to be released into solution. Note, that by ''counterions'' we do not refer here to added salt ions. Ions of added salt will span the entire solution, including the lipoplex interior. Thus, the salt content changes the thermodynamic phase behavior and the value of the adsorption free energy, mainly because a high ambient salt concentration lowers the entropic gain associated with releasing a counterion.

Theoretical predictions and estimates from calorimetry show that for a salt solution of concentration $n^0 = 4$ mM, and a 1:1 CL/HL mole ratio, the gain in free energy upon adsorption at the isoelectric point is a bemusingly large ~7.5 k_BT per fixed charge pair (DNA and CL) (120–122). This value translates to over 2000 k_BT when considering the energy per persistence length of DNA (about 50 nm), carrying approximately 300 charges.

C. Lamellar DNA–lipid Complexes

Many degrees of freedom with competing contributions are expected to ultimately determine the free energy minimum for equilibrium DNA/membrane structures. Typically, these include (but are not limited to) electrostatic energy, elastic bending, solvation, van der Waals, ion mixing, and lipid mixing. Therefore, considering the lipoplex phase behavior, we begin for simplicity by discussing systems where only L_α^c complexes are found. This can be expected when the lipid membranes are rather rigid, such as in the case of mixtures of DOTAP/DOPC (89,102) or DMPC/DC-Chol (123). The main structural parameter for the L_α^c phase is the DNA–DNA distance, reflecting the DNA packing density within the complex. A series of X-ray measurements by Rädler et al. revealed how the DNA–DNA spacings d vary with the ratio ρ of the number of lipid charges to the total number of charges on DNA. The measurements were repeated for each of several different lipid compositions defined by the ratio of charged to overall number of lipids, ϕ. It was found that for a lipid mixture of a given composition ϕ, the spacings are constant throughout the low ρ range where the complex coexists with excess DNA. In the high ρ range, where the complex coexists with excess lipid, the spacings are also nearly constant. Inbetween these limits there exists a ''single-phase'' region, where all the DNA and lipids participate in forming lipoplexes. This region is generally found to include the isoelectric point where, by definition, $\rho = 1$ (Fig. 21).

Several theoretical studies have been proposed to account for this phase behavior (119,124,125). It was found that it is possible to account for most of the experimental observations within the scope of the nonlinear Poisson–Boltzmann equation (125). In this theoretical model, elastic deformations of

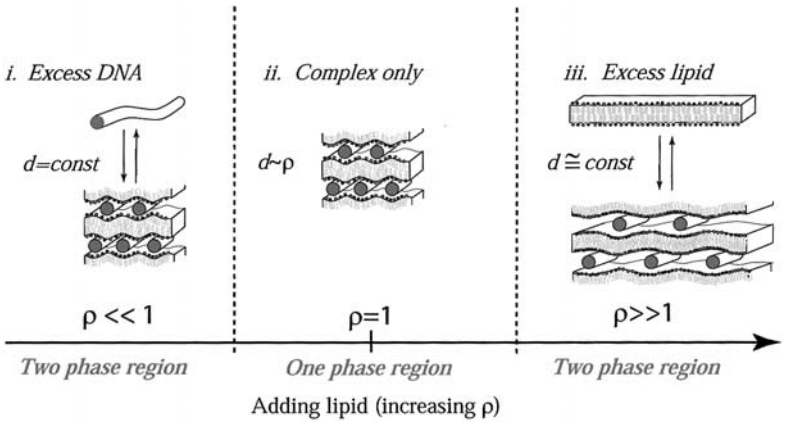


Figure 21 Schematic illustration of the phase evolution of the L_α^c complexes for a constant lipid composition (cationic to nonionic lipid ratio). As lipid is added (ρ increases), the systems evolve from a 2-phase (complex and excess DNA) region through a 1-phase (complex only) region, and finally to a 2-phase (complex and excess lipid) region. The isoelectric point is generally contained within the 1-phase region. See the color insert for a color version of this figure.

the DNA and lipid bilayers were neglected, treating them as rigid macromolecules. On the other hand the lipid's lateral (in plane) mobility in the membrane layer was explicitly taken into account. This turns out to be an important degree of freedom in mixed fluid bilayers, enabling the system to greatly enhance the free energy gain upon complexation, with respect to the case where no lipid mobility is allowed. This adds to the stability of the L_α^c complex. Generally, it was found that lipid mobility favors optimal (local) charge matching of the apposed DNA and lipid membrane. This is the state in which a maximal number of mobile counterions are expelled from the interaction zone, implying a maximal gain in free energy upon complex formation (126). However, the tendency for charge matching (hence migration of lipid to and from the region of proximity) is opposed by an unfavorable local lipid demixing entropy loss. This entropic penalty will somewhat suppress the membrane's tendency to polarize in the vicinity of the DNA molecule. The extent to which the membrane will polarize is determined by the intricate balance between the electrostatic and lipid-mixing entropy contributions to the free energy of the complex. The contribution of lipid demixing to the stabilization of the complex is most pronounced when the membrane's average composition is far from that of the DNA, namely, for low ϕ. Here, the system can gain most out of the polarization so as to come close to local charge matching.

The tendency of charged lipids to segregate in the vicinity of adsorbed rigid macromolecules has gained some experimental support from nuclear magnetic resonance (NMR) studies (127), although many systems may display a more complex behavior. Molecular dynamic simulations of L_α^c complexes, for a lipid mixture of DMTAP and DMPC, showed evidence for a favorable pairing of DMPC and DMTAP lipid molecules through the (partial) negative charge on DOPC, and an interaction of the (remaining) positive charge of the zwiterionic DOPC with the DNA. In contrast to the model discussed above, this implies a nonideal lipid demixing: these lipid molecules preferentially move in pairs (128). This may be anticipated because it is well known that lipids do not generally mix ideally, even in free (unassociated) membranes (129). Furthermore, there is evidence that to some extent neutral lipids also interact directly with DNA (133).

Figure 22 shows the experimental results and theoretical calculations for the dependence of d on ρ for several values of ϕ. For a specific value of ϕ (say $\phi = 0.5$), the 3-phase regimes can clearly be seen. As ρ increases, d changes from ≈ 35 Å (in the excess DNA regime, $\rho << 1$) to ≈ 47 Å (in the excess lipid regime, $\rho >> 1$). Both theory and experiment show that for a wide range of lipid composition, ϕ, there exists a 1-phase, complex-only region at ρ values somewhat larger and smaller than the isoelectric point. This implies that complexes may become either negatively or positively ''overcharged,'' so that the total number of fixed positive and negative charges is not equal. Hence, the complex accommodates either an excess number of lipids or else an excess amount of DNA. The complex's free energy is thus not at its minimum, which occurs at isoelectricity ($\rho = 1$). The interplay between possible phases to minimize the total system's free energy dictates that the complex move away from its minimal free

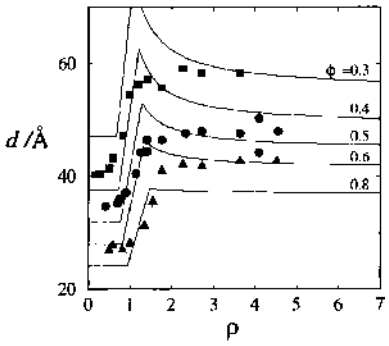


Figure 22 DNA–DNA spacing as a function of ρ in a series of theoretical and experimental results. The theoretical results correspond to (top to bottom) $\phi = 0.3$, 0.4, 0.5, 0.6, and 0.8; all results are presented for a screening length of 50 Å (corresponding to ca. 4 nm of bathing salt solution). The experimental results correspond to $\phi = 0.3$ (squares), 0.5 (circles), and 0.7 (triangles), and were performed with no added salt. (Theoretical results adapted from Refs. (125) and (145); experimental results adapted from Ref. 101.)

energy. The alternative would be to expel the excess lipid ($\rho > 1$) or excess DNA ($\rho < 1$) into solution. The charge densities on these ''free'' unneutralized macromolecules would be very large, rendering this scenario highly unfavorable. Using a simple model based on this overcharging phenomenon, it was possible to account for the considerable extent of this one phase region (125). Within this model, only the uncompensated charges on apposed (DNA–DNA or bilayer–bilayer) surfaces of an L_α^c unit cell (''box'') were considered in estimating the complex's free energy. Figure 22 also shows that as the membrane becomes enriched in CL (ϕ increases) the DNA–DNA distance is systematically reduced, reflecting the fact that smaller amounts of lipid membrane are needed to achieve isoelectricity.

Salt has a significant effect on the phase behavior. In general, added salt causes a significant decrease in d, presumably due to a screening of the repulsive DNA–DNA interaction. This effect is most pronounced when divalent salts are added in increasing amounts. A sharp decrease in the d value is observed for a certain salt molar concentration, resulting in very highly condensed DNA in each gallery (89,130). Another interesting observation is that the identity of the CL's counterion used changes considerably the (endothermic) association enthalpy, particularly in the excess DNA region (121). This probably reflects the nonelectrostatic interaction energies of different ions with membranes, which may influence the thermotropic behavior of the lipid membranes (131,132).

D. DNA Adsorption on Lipid Membranes

Further insight into the in-plane DNA ordering in L_α^c complexes has been gained through the atomic force microscopy

(AFM) study by Fang and Yang (133,134) of DNA adsorption on supported lipid bilayers. In these experiments, DNA was first adsorbed on dipalmitoyldimethylammoniumylpropane (DPDAP) or distearoyl-DAP (DSDAP) CL bilayers, assumed to be in the gel phase. After equilibration and saturation of the surface, the DNA bulk solution was removed, and the surface was put in contact with solution of various concentrations of NaCl. After further equilibration, the salt solution was removed and the surface imaged by AFM. Plasmid and linear DNA similarly treated showed similar results.

Striking, fingerprint-like images of DNA adsorbed on the surface were revealed (Fig. 23). The typical domain size for the aligned, smecticlike order is usually several hundred Angstroms, reflecting the DNA's intrinsic persistence length. These structures are expected to be like those found in L_α^c complexes: the domain size, inferred from x-ray scattering is quite similar (103–104). Furthermore, it was found that the surfaces are often overcharged when DNA is adsorbed, (i.e., the number of DNA fixed charges exceeds the number of lipid charges). This can be anticipated on the basis of theoretical studies of a similar problem: adsorption of charged globular proteins (yet another macroion) on oppositely charged membranes (135). In both cases the driving force for adsorption is similar to that driving lipoplex formation, namely, counterion release. In L_α^c complex formation, much of the DNA can interact with the 2 sandwiching bilayers. In contrast, topology dictates that adsorbates on a single lipid bilayer will always possess a part proximal and a part distal to the interaction zone. If both parts are charged, as is the case with DNA, complete counterion release cannot be achieved because the distal part does not interact significantly with the underlying bilayer. Therefore, although charges on the lipid membrane are fully cancelled by charges on adsorbed DNA macroions, still the portion of DNA away from the contact zone imparts a net surface charge (i.e., overcharging of the DNA-covered membrane).

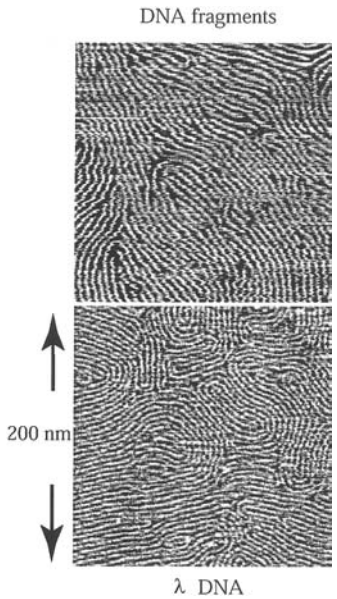


Figure 23 Atomic force microscopy images of DNA from different sources (see figure for details) condensed on DPDAP bilayers at room temperature in 20 mM NaCl. Striking fingerprint-like order is apparent, with a domain size of the order of the persistence length (ca. 50 nm). (Courtesy of J. Yang.)

Yet another interesting feature is the dependence of the DNA–DNA distance on salt concentration. As the NaCl concentration was varied between 20 and 1000 mM, this distance grew from around 45 Å to almost 60 Å. At first this may seem baffling: adding salt should be expected to decrease the DNA–DNA electrostatic repulsion, and hence lower the distance between neighboring interacting strands. This is indeed the general trend that has been observed in L_α^c complexes (101,125). However, because the DNA was primarily allowed to saturate the surface and only subsequently treated with the salt solution (which was later also washed away), adsorption here was not at equilibrium. In fact, when faced with a neat salt solution the adsorbed DNA can only detach, it will not generally readsorb onto the surface. It is therefore hard to give full theoretical reasoning for the trend.

Theoretical explanations have previously been offered to account for this salt-dependent behavior, based on a balance between membrane-mediated effective attraction (that may be the result of the DNA perturbation of the lipid bilayer) and electrostatic repulsion between DNA strands (136). The predicted DNA–DNA spacing as a function of screening length is nonmonotonic: increasing first for low screening lengths and decreasing for high values. An alternative to this approach is related to the free energy gain upon adsorption, and how it changes with the addition of salt. In the presence of added salt, the adsorption free energy can be expected to be lower because the gain in entropy upon release of counterions becomes very small when releasing an ion from an adsorbed layer into a bathing solution with a comparable concentration. Assuming that unbinding would occur when the free energy gain per persistence length is $\approx k_BT$, we can estimate from a simple model that the thickness of the confined layer is $l_{eff} \approx$ 5 Å, rather close to the screening length in solution (3–4 Å) (120–122). Thus, the lower binding free energy may cause some of the DNA strands to dissociate from the lipid surface once the system is exposed to salt. Allowing DNA to rearrange on the surface would then lead to an increase in the average DNA–DNA distance.

When multivalent salt is used, a crowding of DNA molecules is first observed as salt is added (in accordance with the observations in the L_α^c complexes), and then starts to grow for higher concentrations (89,137). This may be a manifestation of the 2 competing forces as salt is added: lessened repulsion between strands vs. weakened adsorption energy.

E. From Lamellar to Hexagonal Complexes

So far, we have discussed the L_α^c lipoplexes formed from lipid membranes that are rigid (bending rigidity much greater than

k_BT) and tend to a planar geometry. Other lipoplex structures may ensue when the lipids possess a spontaneous curvature that is nonplanar, or when the membranes are soft enough to be deformed under the influence of the apposed macroion. The lipid membrane thus responds to the presence of DNA by deforming elastically and by locally changing its composition ϕ.

Membrane elasticity may be varied substantially either by changing the lipid CL/HL composition, changing the lipid species, or by adding other agents, such as alcohols, to the membrane (138,139). In contrast, double-stranded DNA generally remains rather stiff, with a typical persistence length of $\approx$ 500 Å. Hence, the lipoplex geometries are restricted to structures in which DNA remains linear on these large-length scales. Usually, it is the interplay between the elastic (spontaneous curvature and bending rigidity) and electrostatic (charge density) properties of the membrane that will determine the optimum lipoplex geometry at equilibrium.

Often, the membrane elasticity and electrostatic contribution to the free energy display opposing tendencies. For example, the hexagonal H_{II}^c complex, is electrostatically favored due to the cylindrical wrapping of the DNA by the lipid monolayer. This allows better contact between the 2 macromolecular charged surfaces. However, the highly curved lipid geometry may incur a substantial elastic (curvature deformation) energy fee. The price to pay will be lower when the lipid (monolayer's) spontaneous curvature matches closely the DNA intrinsic (negative) curvature or when it has low bending rigidity. Under such conditions, the H_{II}^c complex may become more stable than the L_α^c phase. Usually, a neutral HL is used for adjusting the spontaneous curvature to the required negative curvature because pure CLs typically tend to form uncurved or positively curved aggregates. Use of more HL in the mixed membranes may on the one hand lower the elastic penalty, while on the other hand lower the monolayer's charge density, compromising the electrostatic energy gain upon association.

These qualitative notions were elegantly demonstrated by experiments in which the elastic properties of the lipid monolayers were controlled by changing the nature of the lipid mixture. The spontaneous curvature of the lipid bilayer was modified by changing the identity of HL. It was found that when using a mixture of DOTAP/DOPE, H_{II}^c was the preferred structure, while DOTAP/DOPC mixtures promoted the formation of the L_α^c phase. This is consistent with the fact that pure DOPE forms the inverted hexagonal phase, H_{II}, due to its high negative spontaneous curvature (140–142), while DOPC self-assembles into planar bilayer. In addition, by adding hexanol to the DOTAP/DOPC–DNA lipid mixture, the bending rigidity could be diminished by about 1 order of magnitude (138,139). This induced a clear first order $L_\alpha^c \rightarrow H_{\mathrm{II}}^c$ phase transition (102).

Additional complexity can be expected when accounting for the coexistence of more than 1 phase in solution. A theoretical study of the phase equilibrium took into account the bare lipid phases L_α and H_{II}, the naked DNA and the complex L_α^c and H_{II}^c phases (143). The phase diagram of the system

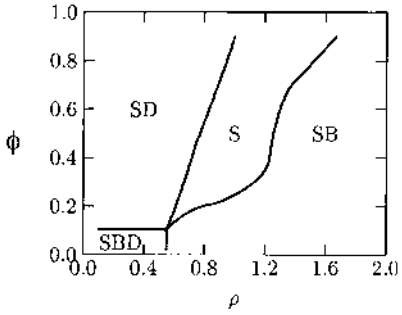


Figure 24 The phase diagram of a lipid–DNA mixture, for lipids that self-assemble into rigid planar membranes. The phase diagram was calculated for membranes characterized by a bending rigidity in the range of $4 < \mathrm{K}_c < \infty\ k_BT$ and a spontaneous curvature of $c = 0\ \mathrm{Å}^{-1}$ for both helper and cationic lipid. The symbols *S*, *B*, and *D* denote, respectively, the L_α^c, L_α, and uncomplexed (naked) DNA phases. (Reprinted by permission from Ref. 143, Biophysical Society.)

was evaluated by minimization of the total free energy, which included electrostatic, elastic, and lipid-demixing contributions. Several systems of different compositions were considered. Figure 24 shows the predicted phase coexistence corresponding to the simplest case already discussed of rigid planar membranes. Results are presented for lipid membranes with a bending rigidity of $\mathrm{K}_c = 10k_BT$ per monolayer and spontaneous curvature $c = 0\ \mathrm{Å}^{-1}$ [typical for many bilayer-forming lipids (106)] for which only lamellar complexes are expected to form. As the overall lipid composition is enriched in CL (higher ϕ) the 1 phase persists over a wider range of ρ. This indicates that for higher CL content, the complex may be expected to be more stable toward addition of either DNA or lipid (hence moving away from the isoelectric point).

The Gibbs phase rule allows for up to three phases to coexist concomitantly for this 3-component (DNA, HL and CL) system. Figure 25 shows the theoretical prediction for the phase diagram for a system in which the HL has a strong negative spontaneous curvature ($\mathrm{K}_c = 10k_BT$ and $c = 1/25\ \mathrm{Å}^{-1}$)(143). For high ϕ values, the phase behavior resembles that of the previously discussed system. However, for lower values of ϕ, a multitude of regions of (up to 3) different phases coexisting together can be found. In some regions, lamellar and hexagonal complexes appear coexisting side by side. A similarly complex diagram results when the membranes are soft (bending rigidity of $\approx k_BT$) as might be expected for membranes with added alcohols (143).

A more subtle demonstration of the underlying balance of forces can be found within the realm of the L_α^c complex. Thus far, the theoretical models considered for the lipid membranes in this lamellar phase assumed them to be perfectly planar slabs. However, this need not be so. When membranes are sufficiently soft (yet not soft enough to favor the H_{II}^c phase) or if one of the CL/HL has a propensity to form curved sur-

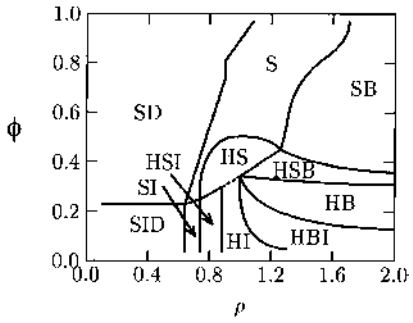


Figure 25 The phase diagram of a lipid–DNA mixture involving ''curvature-loving'' helper lipid. The spontaneous curvature of the helper lipid is $c = -1/25$ Å^{-1}. For the cationic lipid, the spontaneous curvature taken is $c = 0$ Å^{-1}. The bending rigidity for both lipids is $K_c = 10\ k_BT$. The symbols *S*, *H*, *B*, *I*, and *D* denote, respectively, the L_α^c, H_{II}^c, L_α, H_{II}, and uncomplexed (naked) DNA phases. The broken line marks the single H_{II}^c phase. (Reprinted by permission from Ref. 143, Biophysical Society.)

faces, the membrane may corrugate so as to optimize its contact with DNA (see Fig. 19A). If the membrane is further softened, finally, a transition may occur to the H_{II}^c phase. In this respect, the membrane corrugation in the L_α^c complex may be regarded as a further stabilization of the lamellar complex, and a delay to the onset of the $L_\alpha^c \rightarrow H_{II}^c$ transition.

A possible consequence of membrane corrugation in the L_α^c phase is an induced locking between neighboring galleries. This follows the formation of ''troughs'' in a gallery, induced by the interaction of the membrane with DNA in adjacent galleries. This imposes ''adsorption sites'' for the DNA in the 2 neighboring galleries, which propagates the order on. The formation of these troughs, as well as a very weak electrostatic interaction between galleries, may thus correlate between the positions of DNA in different galleries (128,144,145). Limited experimental evidence supports this notion. In cryotransmission electron microscopy (cryo-TEM) studies of the L_α^c phase, spatial correlations were found between DNA strands in different galleries (146). In another series of X-ray studies, the corrugation and charge density modulation in an L_α^c-like complex, in which the membranes are in the gel phase, were measured (147). Further support for the possible formation of corrugations is gained from computer simulations of lipid–DNA complexes (128).

In order to assess the extent of membrane corrugation, a balance of forces between many degrees of freedom should be taken into account. The free energy minimum now depends on the local membrane composition—dictating membrane properties such as local charge density, spontaneous curvature and bending elasticity—and the extent of local deformation around the DNA. Theoretical predictions show that for a wide range of conditions, both stiff and soft membranes can show corrugations that are stable with respect to thermal undulations of the membranes (145). The spacings between galleries and between DNA molecules are also predicted to change somewhat with respect to the case where no corrugations are allowed (144). For the conditions in which the troughs are shallow or absent altogether, one may anticipate the formation of phases where DNA in different galleries are positionally uncorrelated, while orientational order is preserved. These structures were predicted theoretically and termed ''sliding phases'' (103,104,148–150,146).

F. Lipoplex Structure and Transfection Efficiency

In recent years a large number of CL–DNA formulations have been proposed as vectors. However, the fate of the CL–DNA complex once administered, its interaction with the cell membrane, and entry into the cell and subsequently into the cell nucleus, is likely complex and largely unresolved. The poorly understood process of DNA release once in the cell interior must be important (151–153). For example, it has been shown from action in the nucleus that DNA expression is diminished when it is tightly complexed with lipids (156). Hints to the mechanism of the intracellular release of lipoplexes come from experimental evidence in vitro, showing that other added polyelectrolytes may compete with DNA and subsequently replace it in the complex (154). This kind of replacement, by natural polyelectrolytes, may be one way in which DNA is released in cells (155). Another possible mechanism is the fusion of complex lipids with lipid membranes in the cell (89,104).

Only a limited number of experiments have probed the relationship between the structure of CL–DNA complexes and the transfection efficiency. One emergent theme attributes an important role to complex frustration and destabilization in promoting transfection.

Experimental studies show that the 2 ordered complex structures, L_α^c and H_{II}^c, behave differently inside living cells. Furthermore, a correlation was found between the structure of the lipoplexes formed and the transfection efficiency. The structure formed depends in turn on the specific choice and relative amount of HL, CL, and DNA. The H_{II}^c complex was found (in the studied cases) to be a more potent vector than L_α^c (157). Further information is gained from fluorescence studies of cell cultures with both complex types internalized in fibroblast L cells. These indicate that the L_α^c complex is more stable inside the cells, while the H_{II}^c more readily disintegrates—its lipids fusing with the cell's own (endosomal or plasma) membranes—resulting in DNA release. This is in accord with the theoretical findings that the L_α^c complex structure is rather flexible toward changes in the system's compositional parameters, due to its ability to tune both the membrane composition and the DNA–DNA spacing, while this tuning is more limited in the H_{II}^c phase.

The picture is further substantiated by a series of studies by Barenholtz and coworkers (90,152,153,158). In general it was shown that maximal transfection efficiency could be achieved in complexes that were formed in the excess lipid regime (with ρ in the range of 2–5). This correlated well with the point of maximal size heterogeneity of the complexes.

These instabilities were shown to occur concomitantly with an increase in the amount of membrane defects that were in turn mainly attributed to the appearance of several coexisting structures in solution (e.g., H_{II}^{c} and L_{α}^{c} in DOTAP/DOPE lipoplexes, or micellar and lamellar phases in DOSPA/DOPE-based lipoplexes). This is in accordance with the theoretical prediction that the regions of most phase diversity and the largest number of coexisting phases occurs at high ρ (and low ϕ) values (see Fig. 25) (90,114,140).

Other evidence seems to agree with these notions. For example, some successful formulations, such as BIV, are also probably metastable (97,99,110). This may suggest that it is in fact their instability that helps them to release their DNA cargo once they are inside the cell. Attempts have also been made to destabilize lipoplexes more specifically only once they are already internalized in the cells (rather than en route in the serum). Reduction-sensitive cationic lipids were designed, and the subsequent lipoplexes that are formed were shown to undergo large structural changes when exposed to the cytoplasmic reductive systems. The lipoplexes are thus destabilized and the previously packaged DNA is released into the cytosol (92,159–161). A decrease in the toxicity of the CL and increased transfection efficiency are thus achieved (162).

Destabilizing lipoplexes is not the only barrier to transfection. For example, entry of DNA into the nucleus through the nuclear pore complex is inefficient for large pieces of DNA. It has been shown that the cell own nuclear import machinery may be used to increase transfection efficiency dramatically, by attaching a peptide containing a nuclear localization signal (NLS) to the DNA (163,164). Furthermore, the size of the complexes also seems to play a crucial role in determining transfection efficiencies (90,91,97,99). Here, the repulsive interaction between like-surface charge of the complex due to over/undercharging (excess lipid or DNA) can aid in stabilizing the complexes, once they are formed, from fusing further. Another strategy to controlling the interaction between aggregates and the stability of the aggregate in vivo is to modify the composition of the outer wrapping sheath of the lipoplex. The caveat is that the lipoplexes are not stabilized to such a degree that they can no longer disintegrate once inside the cells. For example, short-chain lipids possessing a PEG headgroup (or a derivative thereof) have been used to increase the stability of the lipoplexes in the bloodstream, while not interfering with the endosomal unwrapping once the lipoplexes are internalized in cells (165).

More generally, we can expect that understanding how to control and manipulate the formation of specific phases on the one hand, while better understanding the multistage transfection mechanism and the parameters (conditions) affecting it on the other, should aid in the design of more potent lipid-based gene delivery vectors in the future. These, together with control over the coating and targeting of the complexes, may render these vectors as useful vehicles in gene therapy.

VI. RETROSPECT AND PROSPECT

Structural elucidation of the DNA–cationic lipid complexes and realization of the extent to which they share the structural features of pure DNA or pure lipid polymorphism have advanced notably in the past few years. Some old questions have been answered and new questions raised. It is these new questions that challenge our knowledge of the intricacies of interactions between macromolecules.

The DNA–lipid complexes found so far are only a sample of the much wider set of structures that will be seen on a full DNA–lipid phase diagram. We argue that this larger set of possibilities be approached by firmly established methods to measure the energies of these structures at the same time that they are determined and located on a phase diagram. Built on principles of direct molecular interactions, recognizing the consequences of thermal agitation, this line of observation and analysis can lead to an understanding of the energetic ''whys'' and preparative ''hows'' of complex structures.

Forces so delineated are already knowledgeably applied in new preparations. Precisely how the structure of DNA–lipid aggregates will affect their efficacy in transfection remains to be seen. So far, the ideas we have are too general and have been learned from studying analytically tractable but technically inadequate preparations. General principles do not lead to specific results. Molecules are too interesting to allow easy success in clinical design. Still there is little doubt of a practical link between the energy and structure of these complexes and their viability in a technological application.

Even the present general understanding of forces, even the cartoon ideas of the directions in which forces act in macromolecular complexes can tutor the bench scientist on how to improve preparations. There is enough known for a healthy iteration between experimental attempt and theoretical reason. Experimental successes and failures become the data for molecule force analyses. Various DNA–lipid assemblies reflect the various actions of competing forces. Molecular theorists can define and delineate these forces as they act to create each form; they can provide a logic to design variations in preparation. Basic scientists and clinicians are already in a position to help each other to improve their ways.

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18

Gene Therapy Applications of Ribozymes

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I. INTRODUCTION

Since their discovery in the early 1980s, RNA enzymes or ribozymes have been the subject of much investigation. Numerous studies have been performed to elucidate the biochemistry of how certain RNA molecules can fold into complex tertiary structures to form active sites and perform catalysis. Other studies have focused on identifying the roles that RNA enzymes play in cell biology. More recently, even more attention has been focused on the study of ribozymes because it was recognized that these RNA enzymes can potentially be quite useful for a variety of gene therapy applications.

The first discovered ribozyme was the self-splicing group I intron from *Tetrahymena thermophila*. The reaction mediated by this RNA enzyme has now been extensively characterized and the mechanism by which it excises itself from precursor ribosomal RNAs (pre-rRNA) without the aid of proteins is well understood (1–3). The second ribozyme to be recognized was the RNA subunit of RNase P. RNase P catalyzes the removal of upstream sequences on precursor-tRNAs to produce mature 5′ ends on tRNA molecules in a wide variety of cell types (3,4).

Several other catalytic RNA motifs have been discovered that are naturally associated with plant and human pathogens. The hammerhead and hairpin ribozymes are derived from satellite RNAs from plant viroid and virusoids, and the hepatitis delta virus (HDV) ribozyme is derived from a short single-stranded RNA virus found in some patients with hepatitis B virus. Each of these small RNA enzymes catalyzes a self-cleavage reaction that is believed to play a major role in the replication of these single-stranded RNA pathogens (5).

All these self-cleaving ribozymes have been reengineered so that they can cleave other target RNA molecules in trans in a sequence-specific manner.(6) This ability to specifically cleave targeted RNAs has led to much speculation about the potential utility of trans-cleaving ribozymes as inhibitors of gene expression (7–11). In addition, the group I self-splicing ribozyme from *Tetrahymena* can be reengineered to perform splicing on a targeted RNA molecule in trans. It has been argued that such trans-splicing ribozymes may prove to be effective at repairing mutant cellular transcripts by cleaving off mutant nucleotides and ligating on functional RNA sequences (12–14).

The purpose of this chapter is not to provide an extensive review of the enzymology of ribozymes or to catalog the published results demonstrating that ribozymes may become useful reagents for gene therapy applications. Both of these topics have been extensively reviewed elsewhere (2–14). Moreover, we do not discuss the use of synthetic ribozymes, and leave the description of various gene transfer and expression systems that can be employed to deploy ribozymes to the other chapters in this book. Rather, we attempt to present a focused account of the potential utility of catalytic RNAs for gene therapy by first presenting an overview of the basic biochemistry of well-characterized ribozymes and then discussing how trans-cleaving and trans-splicing ribozymes may be employed for a variety of gene therapy applications. Our hope is that this approach will enhance the reader's understanding of the potential utility of ribozymes for both gene inhibition and genetic repair.

II. CATALYTIC RNAs

Five classes of catalytic RNAs have been extensively characterized. Each class of ribozyme adopts a characteristic secondary and tertiary structure that is required to assemble a catalytic center and perform catalysis (Fig. 1) (6). In addition, these classes of ribozymes differ in size, and the mechanism

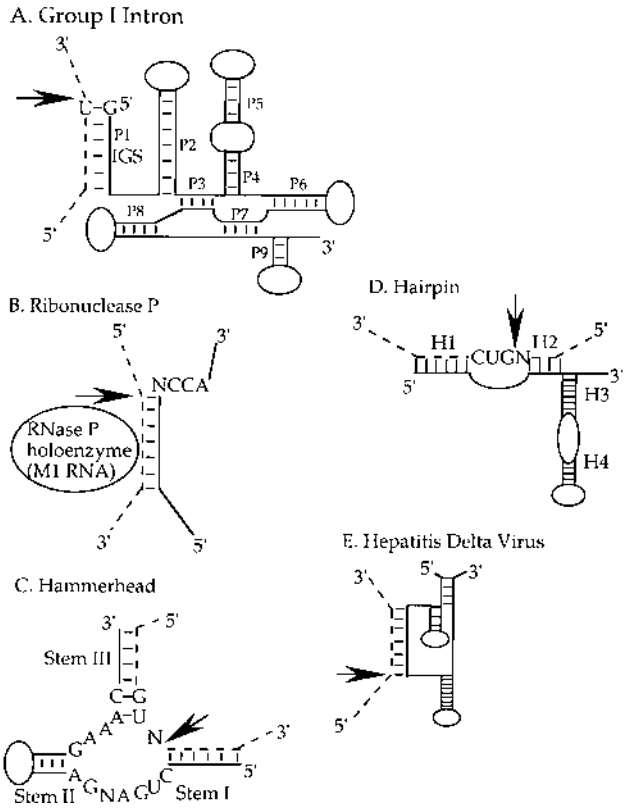

Figure 1 Secondary structures of five classes of ribozymes. All 5 ribozymes, shown in solid black lines, have been engineered to cleave specific target RNAs, shown as dashed lines. The base pairing formed between the ribozymes and their target RNAs are shown and the site of cleavage of the substrate RNAs is indicated by an arrow. (A) The group I ribozyme. P1 through P9 represent the conserved based paired regions found in group I introns. The internal guide sequence (IGS) is shown paired to a target RNA. The substrate is cleaved just 3′ of the conserved G-U wobble base pair. (B) RNase P. RNase P holoenzyme is depicted as a cartoon and contains an RNA subunit as well as a protein cofactor. The substrate for RNase P cleavage is indicated bound to an external guide sequence (EGS) just 5′ of a free 5′-NCCA-3′ sequence. Cleavage of the targeted RNA occurs just across from the end of the EGS-target duplex. (C) The hammerhead ribozyme. The hammerhead ribozyme is shown bound to a target RNA through base pairing interactions formed by stems I and III. The-single-stranded regions encompass the catalytic core of the ribozyme. Cleavage of the substrate RNA occurs at an unpaired residue positioned between stems I and III. (D) The hairpin ribozyme. The hairpin binds its target RNA through 2 base pairing regions called helix 1 (H1) and helix 2 (H2). Cleavage of the target RNA occurs between the N and G nucleotides on the substrate as indicated. (E) The HDV ribozyme. The HDV ribozyme forms 7 or 8 base pairs with its target RNA and cleavage occurs just 5′ of this base pairing interaction.

that each employs to perform catalysis varies. Hammerhead, hairpin, and the HDV ribozymes are only 30 to 80 nucleotides in length and form cleavage products with 2′,3′ cyclic phosphate and 5′-hydroxyl termini. By contrast, catalytic RNAs derived from group I introns and RNase P are typically greater than 200 nucleotides in length and both cleave target RNAs to generate products with 3′-hydroxyl and 5′-phosphate termini. Each class of ribozyme is discussed in more detail below.

A. The Group I Intron from *T. thermophila*

The intervening sequence (IVS) found in nuclear precursor rRNA transcripts from *T. thermophila* is one of the most well-characterized catalytic RNAs. This IVS is a member of a growing family of group I introns that have common structural and functional features. The *Tetrahymena* intron is naturally 413-nucleotides long and is found in the middle of the 26S rRNA gene. The IVS is transcribed as part of the rRNA precursor and excises itself by performing a cleavage and a ligation reaction to form a functional rRNA without the aid of proteins (1,15). Prior to the discovery of this self-splicing reaction, RNA was believed to be only a carrier of genetic information or a scaffold for protein binding and not able to perform catalysis on it own.

B. The Self-splicing Reaction of the *Tetrahymena* Group I Intron

Comparative sequence analysis of several group I introns (16,17) as well as mutational analyses (18–26) have been employed to develop a phylogenetically conserved prediction of the secondary structure of group I introns consisting of a set of paired regions, P1–P9. More recently, X-ray crystallography and chemical probing studies have revealed that the *Tetrahymena* intron adopts a particular 3- dimensional structure using several tertiary interactions (27–34) and contains a catalytic core surrounded by a close-packed layer of RNA helices (32–34). Several studies have shown that this folded RNA structure participates directly in self-splicing (35,36).

The *Tetrahymena* intron excises itself and ligates together its flanking exons by performing 2 consecutive transesterification reactions (2,15). The first step of splicing is a cleavage reaction that is initiated by a free guanosine that is bound by the intron. This guanosine serves as a nucleophile attacking the 5′ splice site and is covalently attached to the 5′ end of the intron (Fig. 2A). The recognition element that defines the exact site of guanosine attack is a G-U wobble base pair that is highly conserved among group I introns. The G-U base pair is part of a short duplex called P1. The P1 duplex includes base pairing between the last 6 nucleotides of the 5′ exon and sequences within the intron called the internal guide sequence (IGS) or the 5′ exon-binding site. In the second step of splicing, the newly generated 3′-hydroxyl group, at the 3′ end of the cleaved 5′ exon, attacks the phosphorous atom at the 3′ splice site, resulting in the ligation of the 5′ and 3′ exons and the excision of

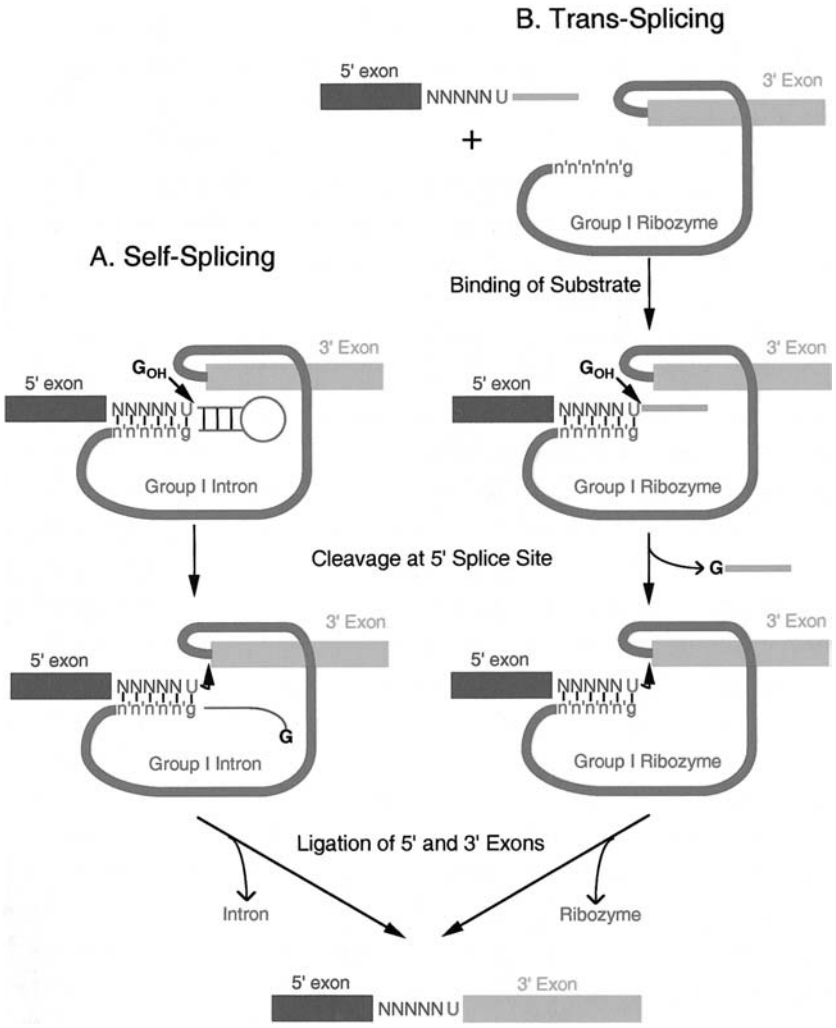

Figure 2 Group I ribozyme-mediated self- and trans-splicing. (A) Self-splicing is initiated by the attack of the 5′ splice site by an intronbound guanosine. This cleavage occurs just 3′ of the uridine shown in red that is involved in a G-U wobble base pair. The group I intron (green) holds onto the 5′ cleavage product via base pairs formed between the internal guide sequence of the ribozyme and the 3′ end of the 5′ exon. In the second step of splicing, the intron attaches the cleaved 5′ exon (blue) onto the 3′ exon (yellow) and liberates itself. (B) During trans-splicing, the ribozyme binds to a sequence in a target RNA (5′-NNNNNU-3′) via base pairing through its internal guide sequence (5′-gn′n′n′n′n′-3′). The ribozyme cleaves the target RNA at the reactive uridine (red), releases the downstream cleavage product (light blue) and ligates a 3′ exon (yellow) onto the upstream cleavage product (dark blue). See the color insert for a color version of this figure.

the intron. The excised group I intron maintains its ability to make and break phosphodiester bonds. However, because the group I intron is not regenerated in its original form following self-splicing, this catalytic RNA is not a true enzyme in the strictest sense (37). Subsequently, shortened forms of the *Tetrahymena* group I intron that lack exon sequences were generated that fulfill the definition of a true enzyme in that they can perform multiple turnover reactions without being modified in the process (38).

C. The Trans-cleaving Reaction of the *Tetrahymena* Ribozyme

Shortened versions of the intervening sequence from *Tetrahymena* that lack the first 19 or 21 nucleotides (called L-19 or L-21) can catalyze the cleavage of oligonucleotide substrates with multiple turnover (38). Moreover, the rate enhancement achieved by this shortened form of the intron is within the range of values achieved by protein enzymes, such as EcoRI,

that catalyze sequence-specific cleavage of nucleic acids. The catalytic mechanism employed by the shortened form of the *Tetrahymena* ribozyme in this trans-cleavage reaction is quite similar to that used by the full-length intron in the cleavage step of self-splicing with a few exceptions (39). First, the 5′ exon sequences preceding the IGS have been removed in the shortened form of the intron. Therefore, the ribozyme must bind to a target RNA that is present in trans (Fig. 2B). As in the case of the self-splicing reaction, a wobble G-U base pair is required at the cleavage site and the IGS of the ribozyme must be complementary to the sequence found on the substrate RNA just 5′ of the reactive uridine residue (40). For the wild-type IGS (5′-GGAGGG-3′), binding would occur at nucleotides 5′-CCCUCU-3′ within the substrate RNA. Cleavage occurs just 3′ of the uridine residue on the substrate RNA at the reconstructed G-U base pair (Fig. 2B).

As noted, substrate recognition and trans-cleavage require base pairing between the IGS on the ribozyme and the RNA substrate. Substrate specificity can be manipulated by altering the sequence of the ribozyme's IGS to make it complementary to any target RNA molecule (40–42). Moreover, no specific sequence requirements exist for the IGS, except that it must contain a guanosine residue at the reaction site. Thus, by altering the guide sequence of the L-21 version of the *Tetrahymena* catalytic RNA, a ribozyme can be created that can be employed to recognize and cleave a target RNA following any uridine residue (Fig. 2B).

The L-21 form of the *Tetrahymena* ribozyme binds to a 6-nucleotide long substrate RNA 10^3- to 10^4-fold tighter than would be predicted by base pairing binding energy alone (43–45). Studies on the L-21 ribozyme, as well as the self-splicing form of the intron, suggest that tertiary interactions contribute to the ribozyme substrate-binding energy. Specific tertiary interactions have been identified that involve 2′-hydroxyl groups on the ribose backbone of the substrate and the intron (46–49). This tight binding between the ribozyme and RNA substrate limits the substrate specificity of the *Tetrahymena* ribozyme because both matched substrates, which are only 6 nucleotides long, and substrates that form single base pair mismatches with the IGS serve as excellent substrates for the ribozyme (44,45). Under conditions of saturating guanosine and 10 mM $MgCl_2$, such RNA substrates are bound so tightly that the ribozyme goes on to cleave essentially every RNA that it binds. Therefore, the substrate specificity of this ribozyme will probably have to be improved if it is to become useful for gene therapy because any 6 nucleotide sequence would be expected to be present in many cellular RNAs. Fortunately, several logical approaches exist that can potentially be employed to enhance the substrate specificity of the *Tetrahymena* ribozyme (50,51).

If the specificity of the group I ribozyme proves very difficult to improve, then trans-cleaving ribozymes derived from group II introns may represent an alternative catalytic RNA motif that may be able to achieve the high levels of substrate specificity that might be required for gene therapy applications. At least some of these group II autocatalytic RNAs, which are present in organelles of plants, lower eukaryotes, and prokaryotes, do not appear to form additional tertiary interactions with their substrates (52). Thus, group II introns may be particularly adept at recognizing only the intended RNA sequence in the pool of cellular transcripts.

D. The Trans-splicing Reaction of the *Tetrahymena* Ribozyme

In addition to performing a trans-cleavage reaction, the *Tetrahymena* ribozyme can also mediate targeted trans-splicing by employing intermolecular cleavage and ligation reactions (41,53). During trans-splicing, a ribozyme with a 3′ exon attached to its 3′ end recognizes a target RNA (5′ exon) by complementary base pairing as in trans-cleavage (Fig. 2B). The ribozyme then cleaves its target RNA as usual at a site immediately 3′ of a conserved G-U base pair formed between a guanosine nucleotide at the 5′ end of the IGS and a uridine nucleotide within the substrate RNA. The sequences downstream of the cleavage site (3′ cleavage product) are then released by the ribozyme. The 3′ end of the 5′ cleavage product is then attached to the 3′ exon, which is originally appended to the ribozyme, to generate the ligated product (Fig. 2B).

As with the trans-cleavage reaction, targeted trans-splicing is very malleable. In principle, any uridine residue in an RNA molecule can be targeted for trans-splicing by simply making the nucleotides in the ribozyme's IGS complementary to the nucleotides that precede an available uridine residue in a targeted RNA. No specific sequence requirements for the 3′ exon are know to exist for this splicing reaction. Thus, essentially any RNA sequence can be employed as a 3′ exon in this reaction and spliced onto a targeted 5′ exon as long as the 3′ exon sequences do not inhibit the ribozyme from folding into a catalytically competent conformation.

E. The RNase P Ribozyme

RNase P, unlike the other 4 ribozymes discussed in this chapter, is the only catalytic RNA that is naturally a true enzyme. RNase P is found in both prokaryotic and eukaryotic cells, where it catalyzes the removal of the 5′ leader sequences from the variety of precursor tRNAs (3,4). This catalytic RNA is naturally part of a ribonucleoprotein (RNP) and in the case of RNase P isolated from *Escherichia coli* the RNP consist of a 377-nucleotide RNA subunit (M1) and a 119-amino acid protein (Fig. 1B). Although it was initially believed that the holoenzyme was required to perform catalysis in vitro, M1 RNA preparations from *E. coli* (54) as well as in vitro-transcribed versions of M1 RNA (55) are able to cleave tRNA precursors with multiple turnover in the presence of high concentrations of magnesium in the test tube. RNase P cleaves substrate RNA by hydrolysis to generate 5′-phosphate and 3′-hydroxyl termini. Although the RNA subunit of RNase P alone is catalytic, the protein cofactor facilitates the RNA processing reaction and allows it to proceed efficiently under physiologically relevant conditions (55).

When processing its natural pre-tRNA substrate, RNase P removes the 5′ leader sequences from the end of the precursor

transcript. Cleavage occurs specifically and accurately just 5′ of the first nucleotide in the mature tRNA, even though only a small degree of sequence conservation exists between different pre-tRNA species. This observation suggested that some facet of the 3-dimensional structure of the precursor tRNAs is the feature of the transcript that is recognized by RNase P. Mutagenesis studies supported this hypothesis because disruption of tRNA folding was shown to decrease the rate of RNase P-mediated cleavage of substrate RNAs. However, the full tertiary structure of tRNA is not required for RNase P recognition and RNA processing (56). Rather the ribozyme appears to recognize a short RNA duplex similar to the acceptor stem of a tRNA just upstream of an unpaired CCA sequence found on the 3′ end of partially processed tRNA transcripts (56). The structure recognized by RNase P can be approximated by a short RNA fragment, termed the external guide sequence or EGS, that is complementary to a substrate (Fig. 1B). RNase P will cleave single-stranded 5′ leader sequences adjacent to any double-stranded RNA duplex as long as the unpaired CCA nucleotides are present at the 3′end of the EGS (Fig. 1B) (56). Thus, through the use of EGS oligonucleotides, RNase P can in principle be targeted to cleave any target RNA.

F. The Hammerhead Ribozyme

The hammerhead ribozyme is a catalytic RNA motif that was originally derived by comparing the self-cleavage domains from a number of naturally occurring viroid and satellite RNAs that replicate in plants (5). The self-cleaving consensus domain consists of a highly conserved catalytic region and 3 helices, and has been shown to have sequence-specific ribonuclease activity (Fig. 1C). Subsequently, a hammerhead domain of less than 60 nucleotides was shown to be sufficient for cleavage (57,58), and two separate oligonucleotides that assembled into a hammerhead structure were shown to mediate a trans-cleavage reaction (59,60).

Crystallographic studies of the hammerhead ribozyme have demonstrated that the tertiary structure of the hammerhead appears to be ''Y shaped'' or like that of a ''wishbone'' (61,62). Helices I and II are in close juxtaposition, whereas helix III is at the bottom of the molecule. However, all 3 are A-form helices. As shown in Fig. 1C, the minimal structural requirements for hammerhead-catalyzed cleavage include two single-stranded regions that contain 9 highly conserved nucleotide sequences, 3 helices, and the nucleotides GUN immediately 5′ of the cleavage site in the substrate RNA. Results of mutagenesis and kinetic studies suggest that the conserved residues comprise the catalytic core of the ribozyme and are required for cleavage (63,64). Helices I and III, which flank the cleavage site, are formed by base pairing with the substrate. This base pairing interaction is extremely important not only because it holds the ribozyme and substrate together, but also because it precisely positions the ribozyme relative to the cleavage site. The most efficient cleavage has been observed with GUC, GUA, or GUU at the cleavage site although some cleavage also occurs after CUC, UUC, and AUC. The hammerhead catalyzes transesterification of the 3′, 5′phosphodiester bond at the cleavage site, which results in the production of RNA with 2′, 3′ cyclic phosphate and 5′-hydroxyl termini.

Much effort has been focused on developing hammerhead ribozymes into useful therapeutic agents. The hammerhead's small size and simple secondary structure, containing helices I and III, which can be made to base pair with virtually any substrate RNA, has allowed a great number of investigators to design hammerhead ribozymes to target any RNA molecule for cleavage and destruction (discussed in detail below). In particular, efforts have been directed at optimizing the interaction between the ribozyme and its substrate because both the length and base composition of complementary helices I and III can affect substrate specificity, ribozyme substrate affinity, and rate of reaction turnover.

G. The Hairpin Ribozyme

As is the case for the hammerhead ribozyme, the hairpin ribozyme represents a catalytic RNA motif that is derived from RNA associated with a plant pathogen (5). This small catalytic RNA was discovered in the 359-nucleotide long negative strand satellite RNA of Tobacco Ringspot virus [(−)sTRSV], which was shown to mediate a self-catalyzed cleavage reaction as part of its replication pathway (57,65).

A minimal catalytic domain for this RNA molecule has been identified (66,67), which consists of a 50-base RNA catalyst that efficiently cleaves an RNA substrate containing 14 bases of satellite RNA sequence (68). Features of secondary structure within this domain, defined from minimum energy RNA folding calculations and supported by mutagenesis studies, include 4 helices, 2 of which are formed by base pairing between the RNA catalyst and the substrate (Fig. 1D) (69). These 2 helices form part of the substrate recognition site and flank a 4-base loop within the substrate (5′-AGUC-3′) containing the cleavage site. For the wild-type (−)sTRSV, cleavage occurs between the nucleotides, A and G, by transesterification generating a 5′ fragment with 2′, 3′ cyclic phosphate termini and a 3′ fragment with a 5′-hydroxyl termini. Trans-cleavage has also been observed in vitro with multiple turnover when substrate RNAs are added to the hairpin ribozyme as separate transcripts (68,69).

The hairpin catalytic RNA motif can be designed to target a great variety of RNA molecules for cleavage because only 2 sequence requirements exist for this reaction. First, to maintain catalytic activity complementary base pairing between the ribozyme and substrate must occur. Single base pair mismatches at the 10 positions included in the 2 flanking helices can result in the loss of catalytic activity (69), although single base pair mismatches distal to the cleavage site appear to be tolerated (70). In addition, it has been noted that the composition of base paired substitutions in these helices can have a wide range of affects on the kinetic properties of the ribozyme (69), suggesting that base pair substitutions should be optimized for each application. The second sequence requirement for the hairpin cleavage reaction involves the nucleotides that compose the target site. Optimal substrate cleavage occurs with the nucleotides GUC immediately 3′ of the cleavage site.

The guanosine residue appears to be essential and is believed to be directly involved in catalysis (71). Moreover, catalytic activity has been shown to vary widely when nucleotide substitutions are made at the other 3 positions (71).

H. The HDV Ribozyme

HDV is a 1700-nucleotide, covalently closed circular RNA that is associated with hepatitis B virus infection in certain patients. This animal RNA virus undergoes autocatalytic self-cleavage as part of its replication cycle (5). A minimal self-cleaving RNA motif have been determined for the HDV catalytic RNA (72,73), which includes approximately 85 nucleotides from both the genomic (74) and antigenomic RNAs (75). Features of secondary structure were proposed based on nuclease probing and/or site-directed mutagenesis, and were found to be similar for both the genomic and antigenomic self-cleaving sequence elements (75). More recently, X-ray crystal structure studies have been employed to solve the 3-dimensional structure of the genomic HDV ribozyme (76). Such analysis demonstrates that the ribozyme forms 4 stems, 2 of which (stems I and II) generate a tertiary interaction called a pseudoknot (Fig. 1E) (75,76). Stems II, III, and IV appear to be important for stabilizing the catalytic form of the ribozyme, whereas formation of stem I is required for efficient cleavage (75).

The self-cleavage reaction mediated by the HDV ribozyme occurs by transesterification and is dependent on divalent cations. Cleavage products have 5′-hydroxyl and 2′,3′ cyclic phosphate termini. The rates of HDV self-cleavage in vitro using either in vitro-transcribed HDV RNA or HDV RNA isolated from infected tissue appears to be very slow. However, addition of urea or formamide has been shown to increase the rate of cleavage as much as 50-fold, suggesting that these denaturants may be mimicking a viral or cellular RNA-binding or unwinding factor that facilitates cleavage in vivo (77). A derivative of the catalytic RNA motif from HDV has been engineered to catalyze cleavage reactions in *trans* with multiple turn over (78,79). Target site recognition is dependent on the formation of 7 to 8 base pairs formed between the target RNA and the HDV ribozyme 3′ of the cleavage site (79).

III. THERAPEUTIC APPLICATIONS OF TRANS-CLEAVING AND TRANS-SPLICING RIBOZYMES

Ribozymes have the potential to become useful therapeutic agents, and currently they are being developed for a wide variety of clinical applications. The vast majority of effort has been expended in the development of trans-cleaving hammerhead and hairpin ribozymes as inhibitors of viral gene expression. In particular, these ribozymes have been targeted to cleave and destroy HIV-1 RNAs to inhibit viral replication in infected cells. The effectiveness of ribozymes as inhibitors of cellular gene expression in both prokaryotes and eukaryotes has been examined, and in a number of studies hammerhead ribozymes have been used to reveal particular gene functions. Trans-cleaving ribozymes are also being developed to target transcripts encoding oncogenes, such as the *bcr/abl* and c-Ha-*ras*. As mentioned earlier, new ribozyme applications are being developed for the group I trans-splicing ribozyme from *T. thermophila*, which can be used to repair defective cellular RNAs and to revise viral transcripts to give them antiviral activity. In the following section, we discuss the therapeutic applications of trans-cleaving and trans-splicing ribozymes. Because so much effort has been devoted to developing trans-cleaving ribozymes that target HIV-1 RNA, a more a detailed summary of the work performed in this area is presented.

A. Inhibition of Gene Expression by Trans-cleaving Ribozymes

1. Inhibition of HIV Replication by Ribozymes

Several different approaches have been described that employ RNA molecules to render cells resistant to HIV replication, including the use of antisense RNA and RNA decoys (80). Although these and other approaches have been reported to be effective in suppressing HIV replication in infected cells, ribozymes have 2 theoretical advantages as compared with other RNA-based HIV inhibition strategies: (1) cleavage of viral transcripts results in the direct, irreversible inactivation of the target RNA; and (2) fewer ribozymes may be required to inhibit a given target gene effectively because a single ribozyme can catalyze multiple cleavage reactions and thus destroy multiple viral transcripts. However, like many other antivirals, ribozymes may also be sensitive to HIV sequence heterogeneity and effective inhibition may require the use of a combination of these strategies.

Ribozymes may be able to cleave viral target RNAs at a number of stages in the viral life cycle. Potential RNA targets include incoming genomic RNAs, early viral mRNAs, late viral mRNAs, and full-length genomic RNAs that are being encapsidated into virion (Fig. 3). Although cleavage of incoming RNAs would prevent viral integration and therefore be highly effective in protecting cells, the fact that HIV genomic RNAs are encapsidated within a viral core may make these transcripts difficult to access by ribozymes. Moreover, the viral polymerase may initiate reverse transcription before the ribozyme can base pair with and cleave the target sequence, setting up a race between the ribozyme and the reverse transcriptase machinery. Nevertheless, several reports suggest that ribozymes may be able to inhibit the initial step of the viral life cycle by cleaving incoming HIV genomic RNAs. However, in most these HIV inhibition studies, cleavage of incoming genomic RNAs has been assessed only semiquantitatively. Differences in the amount of proviral DNA (81) or gag mRNA (82) in ribozyme-expressing cells and controls cells has been only analyzed by PCR amplification reactions, which were not internally controlled. Unfortunately, no system has been developed to date that allows for the direct detection of cleavage products of incoming HIV genomic RNAs in mammalian cells.

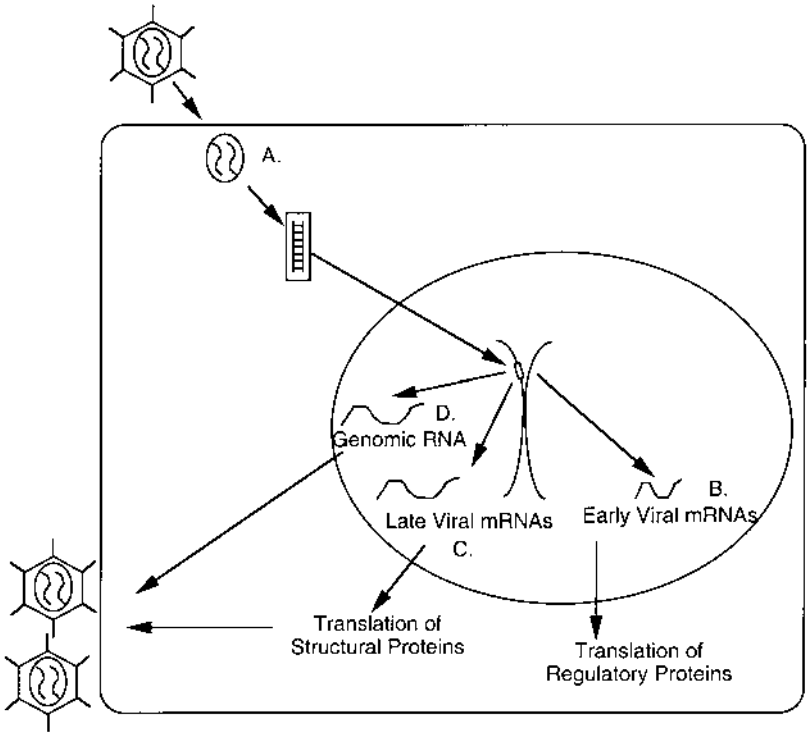

Figure 3 HIV RNAs in the context of the viral life cycle. Trans-cleaving ribozymes can potentially inhibit HIV replication by cleaving and destroying viral RNA at a number of steps in the HIV life cycle. (A) Viral genomic RNAs can be targeted prior to reverse transcription into dsDNA and proviral integration. (B) During early gene expression, messenger RNAs encoding regulatory proteins are made. (C) During late gene expression, mRNAs encoding structural proteins are produced. (D) Full-length genomic RNAs are expressed for packaging into viral particles budding from the cell surface.

Early viral transcripts may prove to be the most attractive targets for conferring resistance to HIV-1 (Fig. 3). Viral RNAs expressed at this stage of the HIV-1 life cycle, such as those encoding tat, rev, and nef proteins, are not very abundant. Thus, fewer catalytic RNAs may be required to be protective. Cleavage of early transcripts that inhibit the expression of regulatory proteins such as rev should also result in the inhibition late gene expression.

Ribozyme-mediated cleavage of late viral transcripts may not be effective in inhibiting HIV replication. Although these RNAs may be accessible for ribozyme cleavage, their shear abundance would probably require that extremely high levels of ribozyme be expressed to reduce their levels in infected cells. In addition, the detrimental affects mediated by early viral regulatory proteins would not be inhibited, even if late viral gene function was eliminated. An alternative strategy may be to target highly conserved sequences in the long terminal repeats present in all viral RNAs. This approach could result in the inhibition of both early and late gene expression.

As mentioned above, much work has been performed to develop ribozymes for HIV gene therapy strategies. Hammerhead and hairpin ribozymes are particularly well suited for this purpose because of their small size, simple secondary structure, and the ease with which they can be manipulated to target specific HIV substrate RNAs for cleavage. In the first application of this approach to inhibit HIV replication, an anti-*gag* hammerhead ribozyme was generated that specifically cleaved gag-encoding RNAs in vitro and inhibited HIV-1 replication in a human T cell line (82). Subsequently, such trans-cleaving ribozymes have been designed to target a variety of highly conserved sequences throughout the HIV-1 genome and have been shown to inhibit HIV replication to varying degrees in a number of tissue culture studies [for extensive review, see (83,84)]. Moreover, certain trans-cleaving ribozymes have been shown to be able to inhibit the replication of diverse viral strains as well as clinical isolates in primary T cell cultures (81,83–85). Comparisons between catalytically active and inactive forms of these anti-HIV ribozymes have demonstrated that maximal inhibition of virus replication is usually associated with catalytic activity and not simply due to the antisense property of these anti-HIV ribozymes (86–88).

To assess the activity of ribozymes in more clinically relevant settings, human peripheral blood lymphocytes have been stably transduced with a hairpin ribozyme targeting U5 region

of the HIV-1 genome. These cells were shown to resist challenge by both HIV-1 molecular clones and clinical isolates (89). More recently, macrophage-like cells, which were differentiated from hematopoietic stem/progenitor cells from fetal cord blood and which were stably transduced with a hairpin ribozyme targeted at the 5′ leader sequence, resisted infection by a macrophage-tropic virus (90). Transduction of pluripotent hematopoietic stem cells with HIV-resistance genes may represent an avenue to continually generate cells that are resistant to HIV infection. Such stem cells differentiate into monocytes and macrophages, the major targets of HIV-1 infection.

The generation of sequence variants during HIV-1 replication has posed a major problem for immunization strategies and anti-HIV-1 drug therapies designed to suppress viral replication in infected patients. Frequent substitution of amino acids within the variable domains of the HIV-1 *env* gene has resulted in the emergence of neutralization escape mutants both in cell culture and in vivo. Similarly, the rapid emergence of resistant viral strains has limited the effectiveness of both nucleoside and nonnucleoside analog reverse transcriptase inhibitors. Selection for variants resistant to anti-HIV-1 ribozymes is also likely to occur because a single-point mutation at the cleavage site on the substrate RNA could inhibit ribozyme-mediated cleavage of viral transcripts. Unlike small molecule drugs that require substitutions at the protein level, even a silent-point mutation can generate a ribozyme-resistant strain. The affect of single-point mutations at the cleavage site of hairpin (5′N/\GHY3′, where H = U, C, or A, and Y = C or U) and hammerhead (5′NUX/\3′, where X = C, U, or A) ribozymes has not been studied, but the more stringent sequence requirement at the cleavage site of the hairpin ribozyme could enhance its sensitivity to mutations. Approaches that have been suggested to overcome sequence heterogeneity among HIV isolates include the development of multitargeted ribozymes that cleave a given RNA at multiple sites and to target either single or multiple ribozymes to highly conserved sequences within the HIV genome.

In summary, several studies have suggested that ribozymes can inhibit HIV replication in cell culture experiments when cells are challenged with very low innoculums of HIV. It remains to be tested if this first generation of ribozymes can also inhibit virus replication in HIV-infected patients under conditions of active viremia and where a multitude of quasispecies of the virus preexist. Ultimately, this question will be answered as ribozymes begin to be evaluated in clinical trials in HIV-infected individuals (91–93).

2. Inhibition of Hepatitis C Virus by Ribozymes

In addition to its use as an anti-HIV-1 antiviral, hammerhead ribozymes are also being used to target hepatitis C virus (HCV) infection (94). Fifteen different hammerhead ribozymes were designed to target different sites in the conserved 5′ untranslated region (UTR) present in all HCV RNAs, and tested for their ability to reduce expression of a luciferase reporter gene downstream of the HCV 5′ UTR. Cotransfection of OST7 cells with target and ribozyme plasmids resulted in a 40% to 80% reduction in luciferase activity. In a more realistic setting, inhibition of polio viral replication in HeLa cells was ascertained by a chimeric construct that contains the HCV 5′UTR fused to the polio virus. Several HCV 5′ UTR targeted ribozymes inhibited the chimeric HCV-PV replication by 90%, suggesting that hammerhead ribozymes may be useful in reducing viral burden in HCV infection.

3. Inhibition of Tumor Growth and Metastasis by Ribozymes

The use of hammerhead ribozymes targeted to vascular endothelial growth factor (VEGF) receptors was investigated to prevent angiogenesis and subsequent tumor growth and metastasis in vivo (95). Ribozymes targeted to Flt-1 (VEGF-1) and KDR (VEGF-2) receptors reduced tumor growth in a metastatic variant of Lewis lung carcinoma, but only the ribozyme targeted to Flt-1 inhibited lung metastasis. In addition, ribozyme inhibition of Flt-1 mRNA expression also reduced liver metastasis in a human metastatic colorectal cancer model. These data suggest that ribozyme inhibition of gene expression in vivo is possible and that inhibition of VEGF receptor expression can reduce tumor growth and metastasis.

B. Trans-cleavage of mRNAs Encoding Dominant Oncogenes

Neoplastic transformation is often associated with the expression of mutant oncogenes. Because ribozymes can be designed to inhibit the expression of specific gene products, their potential as antineoplastic agents is currently being evaluated. For example, hammerhead ribozymes have been reported to be able to suppress the tumorigenic properties of cells harboring an activated human *ras* gene (96–98). More recently, the *bcr/abl* fusion transcript has been the target of many ribozyme studies (99–102). This abnormal mRNA is transcribed from the Philadelphia chromosome, which is present in 95% of patients with chronic myelogenous leukemia (CML) and in many patients with acute lymphocytic leukemia (ALL). In vitro experiments have shown that the 8500-nucleotide long *bcr/abl* transcript was efficiently cleaved by an anti-*bcr/abl* ribozyme. In CML blast crisis cell lines, expression of ribozymes targeted at *bcr/abl* mRNA was reported to be able to reduce the production of $p210^{bcr/abl}$, and *bcr/abl* transcripts and reduce cell proliferation.

C. RNA Revision by Trans-splicing Ribozymes

Recently, it has been argued that ribozymes may also be able to alter the sequence of targeted RNAs not just destroy them and that such RNA revision may be useful for treating a variety of diseases via gene therapy (12). As described earlier, the *Tetrahymena* group I ribozyme can catalyze a trans-splicing reaction (20,53). Such targeted trans-splicing can potentially be used to repair mutant transcripts and to alter viral RNAs to give them antiviral activity.

In the targeted trans-splicing reaction, the *Tetrahymena* ribozyme recognizes and binds to its substrate RNA (the 5′ exon) by base pairing between the IGS and a sequence in the substrate. Following cleavage, the ribozyme splices its 3′ exon onto the cleaved substrate RNA (Fig. 2c). Because the ribozyme cleaves after the sequence N_5U, the only sequence requirement for the substrate is to have a uridine residue preceding the cleavage site. Thus, any uridine nucleotide in an RNA molecule can in principle serve as a target for the ribozyme if the target sequence is accessible for ribozyme binding. Moreover, because there are no sequence requirements for the 3′ exon in the trans-splicing reaction (2), almost any sequence can be spliced onto the 5′ target transcript.

The lack of sequence requirements for the 3′ exon suggests that it could be manipulated so that trans-splicing can be employed to replace a defective portion of an RNA transcript with a functional sequence (Fig. 4). Trans-splicing ribozymes could be designed that would cleave defective transcripts upstream of point mutations or small insertions or deletions. A 3′ exon consisting of the wild-type sequence could then be spliced onto the cleaved target, resulting in a corrected mRNA. Trans-splicing ribozymes can be employed to repair defective RNA messages. In the first example of this application, the group I ribozyme from *T. thermophila* was reengineered to repair truncated *lacZ* transcripts via targeted trans-splicing in *E. coli* (103) and in mammalian cells (104). In both settings, the ribozyme was shown to be able to splice restorative sequences onto mutant *lacZ* target RNAs with high fidelity and thus maintain the open reading frame for translation of the repaired transcripts. In a subsequent study, the efficiency of RNA repair was monitored and the ribozyme was shown to be able to revise up to 50% of the truncated *lacZ* transcripts when ribozyme and *lacZ* substrate- encoding plasmids were cotransfected into mammalian fibroblasts (105).

More recently, several groups have demonstrated that group I ribozymes can be employed to amend faulty transcripts that are associated with common genetic diseases. Phylactou et al. demonstrated that a trans-splicing ribozyme could be employed to amend transcripts associated with myotonic dystrophy (106), Lan et al. employed this RNA repair approach to correct mRNAs associated with sickle-cell disease (Fig. 4) (107), Watanabe et al. corrected mutant p53 transcripts,(108) and Rogers et al. repaired mutant canine skeletal muscle chloride channel (109). In the myotonic dystrophy case, a trans-splicing ribozyme was employed to shorten the trinucleotide repeat expansion found in the 3′ untranslated region of the human myotonic dystrophy protein kinase transcript in cell culture studies (106). In the sickle-cell experiments, trans-splicing was employed to convert sickle β-globin transcripts into γ-globin-encoding mRNAs in erythrocyte precursors isolated from patients with sickle-cell disease (107). In both studies, sequence analysis of the amended RNAs demonstrated that the ribozyme had accomplished such repair with high fidelity, forming the proper splice junctions between the targeted transcript and the corrective sequences. In addition, a trans-splicing ribozyme was employed to repair mutant p53 in human cancer cells. After trans-splicing, repaired functional p53 was monitored by the induction of luciferase gene under the control of a p53-dependent promoter (108). These data suggest that ribozyme repaired mRNAs are translated in mammalian cells and retain wild-type activity. Finally, Rogers et. al. used a trans-splicing ribozyme to correct a mutant canine skeletal muscle chloride channel (cCIC-1). Although repair efficiency was low (1.2%) when ascertained by quantitative reverse transcription and polymerase chain reaction (RT-PCR) in a population of cells, patch-clamp analysis of individual cells yielded a wide range of repair efficiency with 18% of cells showing some restoration and several cells showing complete restoration of wild-type function (109).

Trans-splicing ribozymes have recently been employed to deliver new gene activities to *E. coli* (110), yeast (111), and mammalian cells (112). Kohler et al. (110) designed a trans-splicing ribozymes that contain a 3′ LacZ exon and targets either chloramphenicol acetyltransferase, HIV-1, or cucumber mosaic virus sequences. After induction of target and ribozyme expression in *E. coli*, trans-splicing was monitored by LacZ expression. In addition, Ayre et al. (111) investigated the utility of using a ribozyme that targets the coat protein of cucumber mosaic virus to inhibit viral propagation while selectively destroying virally infected cells. A trans-splicing ribozyme was developed that contains the diptheria toxin A chain (DTA) as a 3′ exon, which is spliced in frame to the viral target sequence. After trans-splicing, yeast cells that express the spliced viral target RNA fail to grow. Finally, this new genetic approach was developed to combat HVC infection in human cells (112). A trans-splicing ribozyme was targeted to a site in the HCV internal ribosome entry site (IRES) present in all viral RNAs and used for cap-independent translation of viral genes in mammalian cells. The 3′ exon contained the IRES sequence after the splice site fused in-frame to the DTA. After trans-splicing and IRES-mediated translation of the DTA, virally infected cells were destroyed by apoptosis. These data suggest that trans-splicing ribozymes can be used specifically and selectively to deliver new gene activities to a variety of cell types.

These results demonstrate that a trans-splicing group I ribozyme can be employed to repair pathogenic transcripts in clinically relevant, cellular settings. However, as with the development of almost every novel therapeutic approach, several technical issues must be addressed before ribozyme-mediated repair of mutant RNAs can become useful in the clinic. First, it remains to be determined whether repair of any pathogenic transcript can proceed efficiently enough in primary human cells to be therapeutically beneficial. In the case of sickle-cell disease, conversion of as little as 5% to 10% of the sickle β-globin transcripts into mRNAs encoding γ-globin is expected to greatly reduce cell sickling and thus the severity of the disease. Whether this relatively modest level of repair can be achieved in erythrocyte precursors from individuals with sickle cell disease is unclear but results demonstrating that 50% of the mutant *lacZ* transcripts expressed in mammalian cells can be revised by ribozymes (105) is at least encouraging in this regard. Second, the specificity of trans-splicing may have to be increased because in mammalian cell experiments the *Tetrahymena*

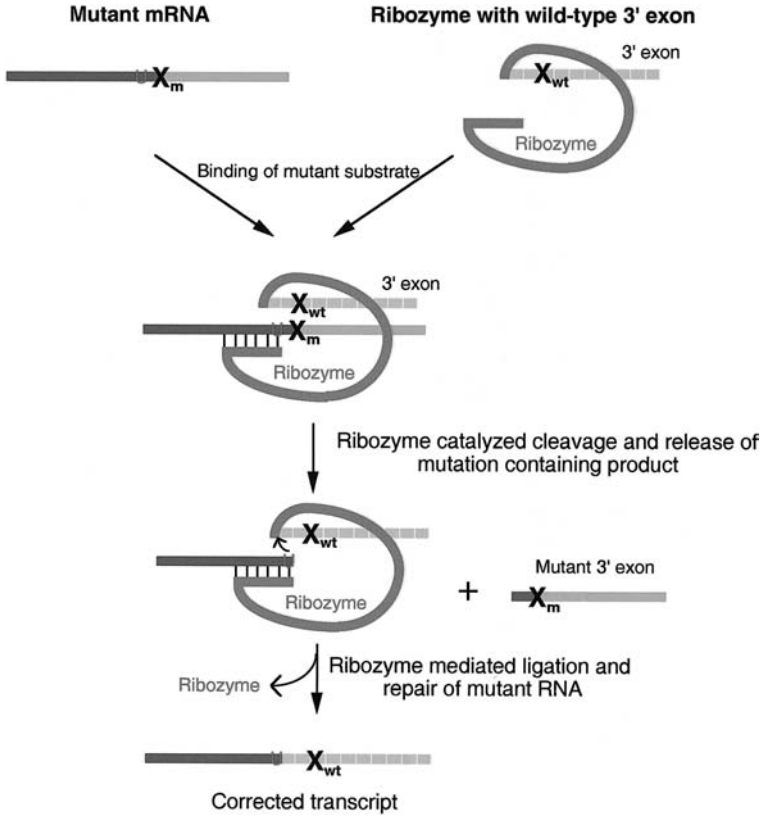

Figure 4 Ribozyme-mediated repair of mutant transcripts. A trans-splicing ribozyme (green) binds to a mutant RNA transcript that contains a point mutation, deletion, or insertion. The IGS on the ribozyme recognizes a uridine residue (red) 5′ of the mutation (indicated by Xm). The ribozyme cleaves the mutant RNA and releases the downstream, mutation-containing cleavage product (light blue). Next, the ribozyme ligates the wild-type sequence (Xwt and yellow) of the target RNA onto the upstream cleavage product to yield a repaired transcript. See the color insert for a color version of this figure.

group I ribozyme was shown to react not only with intended *lacZ* target RNAs, but also with other cellular transcripts (104). Such limited reaction specificity is fully anticipated from knowledge about the energetics of substrate binding by this ribozyme. This biochemical knowledge is now being used to redesign the ribozyme to enhance its specificity (51).

In summary, the ability to employ trans-splicing ribozymes to revise genetic instructions embedded in targeted RNAs represents a broad new approach to genetic therapy. Because defective RNAs can only be repaired in the cells in which they are present and only when they are expressed, RNA repair may become an effective means of recapitulating the natural expression pattern of therapeutic genes. Moreover, RNA repair may be especially useful in the treatment of genetic disorders that are associated with the expression of dominant or deleterious mutant RNAs and proteins. In these cases, RNA repair should simultaneously engender wild-type protein production and eliminate production of the deleterious gene product. For these reasons, the concept of RNA repair is likely to continue to attract increased interest from gene therapists.

IV. CONCLUSION

Trans-cleaving and trans-splicing ribozymes either alone or in combination with other therapeutic agents have the potential to restore genetic information or to eliminate it. The information presented in this chapter describes how ribozymes can be employed to mediate repair of defective transcripts or the destruction of pathogenic RNAs. Although the results from the first generation of therapeutic ribozyme experiments are in general quite encouraging, the long-term utility of catalytic RNAs is still unclear. Many factors may limit the efficacy of ribozymes in the clinic. Thus, it will be essential to evaluate RNA catalysis in clinically relevant settings and to use knowledge gained from such experiments to aid in the design of therapeutic ribozymes. Once adequately developed, catalytic RNAs should become new and useful weapons in the wars being waged on a great number of devastating diseases.

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19

Antisense Oligonucleotide-based Therapeutics

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I. INTRODUCTION

Antisense oligonucleotides are short synthetic oligonucleotides, usually between 15 and 25 bases in length designed to hybridize to RNA through Watson–Crick base pairing (Fig. 1). Upon binding to the target RNA, the oligonucleotide prevents expression of the encoded protein product in a sequence-specific manner. As the rules for Watson-Crick base pairing are well characterized (1), antisense oligonucleotides represent, in principal, a simple method for rationally designing drugs. In practice, exploitation of antisense oligonucleotides for therapies has presented a unique set of challenges, some anticipated and others unanticipated. Nevertheless, antisense oligonucleotides are showing promise as therapeutic agents broadly applicable for the treatment of human diseases. Currently, there is 1 approved antisense product in the market and at least 20 agents currently in clinical trials, several of which are in advanced stages of development (Table 1). In this chapter, we summarize the properties of antisense oligonucleotides in terms of their application as therapeutic agents. As expected, there is significantly more information regarding first-generation phosphorothioate oligodeoxynucleotides; this serves as a good benchmark for comparison with some of the newer modified oligonucleotides. One antisense mechanism that we do not discuss in this chapter are ribozymes, as they are covered elsewhere in this volume.

II. ANTISENSE MECHANISM OF ACTION

Antisense oligonucleotides are small synthetic oligonucleotides that are designed to bind to mRNA through Watson–Crick hybridization. Upon binding to the RNA, the oligonucleotide may inhibit expression of the encoded gene product through either inducing cleavage of the RNA by RNases such as RNase H or by occupancy of critical regulatory sites on the RNA (Fig. 2). Several studies have documented that phosphorothioate oligodeoxynucleotides promote cleavage of the targeted RNA by a mechanism consistent with RNase H cleavage (2–6). RNase H is a ubiquitously expressed enzyme that cleaves the RNA strand of an RNA–DNA heteroduplex (6,7). If the antisense oligonucleotide use DNA chemistry, it will direct RNase H to specifically cleave the target RNA upon binding.

Another RNase-dependent antisense mechanism that has recently received much attention is interference RNA or RNAi (8–13). Introduction of long double-stranded RNA (dsRNA) into eukaryotic cells leads to the sequence-specific degradation of homologous gene transcripts. The long dsRNA molecules are metabolized to small 21 to 23 nucleotide interfering RNAs (siRNAs) by the action of an endogenous ribonuclease, Dicer (14–16). The siRNA molecules bind to a protein complex, termed RNA-induced silencing complex (RISC), which contains a helicase activity that unwinds the 2 strands of RNA molecules, allowing the antisense strand to bind to the targeted RNA molecule (12,17). The RISC is also believed to contain an endonuclease activity, which hydrolyzes the target RNA at the site where the antisense strand is bound. It is unknown whether the antisense RNA molecule is also hydrolyzed or recycles and binds to another RNA molecule. Therefore, RNA interference is an antisense mechanism of action, as ultimately a single- strand RNA molecule binds to the target RNA molecule by Watson–Crick base pairing rules and recruits a ribonuclease that degrades the target RNA.

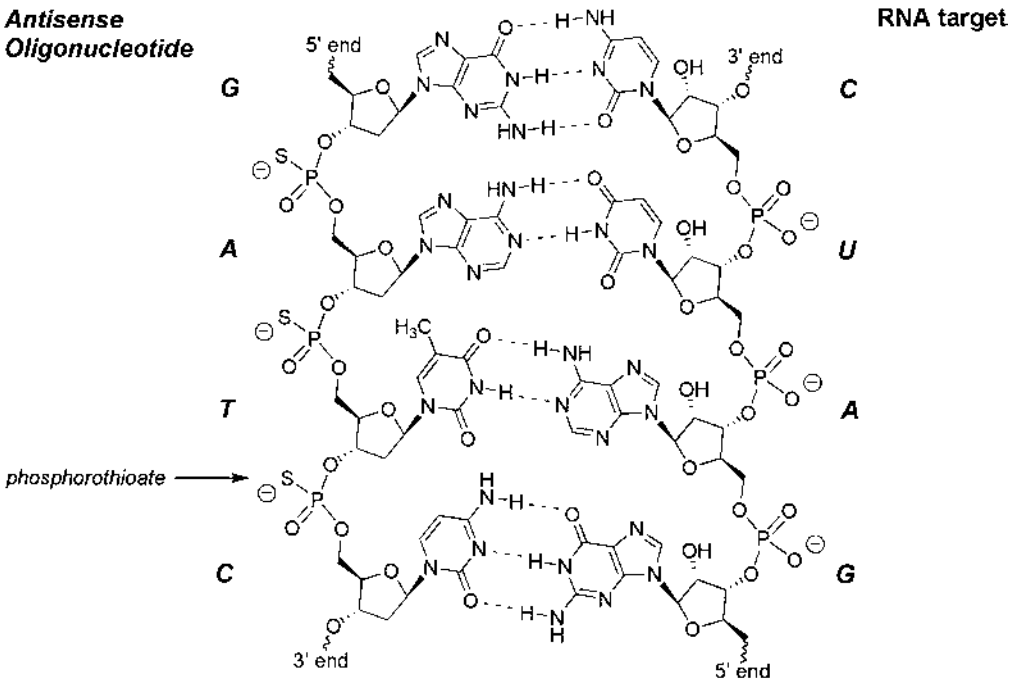

Figure 1 Phosphorothioate antisense oligodeoxynucleotide targeting an RNA receptor. Watson–Crick base pairing rules are indicated: nucleobase adenosine hydrogen bonds to nucleobase uracil, nucleobase cytosine hydrogen bonds to nucleobase guanine.

In mammalian cells, long double-stranded RNA molecules were found to promote a global change in gene expression, obscuring any gene-specific silencing (18,19). This reduction in global gene expression is believed to be mediated in part, through activation of double-stranded RNA-activated protein kinase (PKR), which phosphorylates and inactivates the translation factor eIF2α (20). Recently, it has been shown that transfection of synthetic 21-nucleotide siRNA duplexes into mammalian cells does not elicit the PKR response, allowing effective inhibition of endogenous genes in a sequence-specific manner (21,22). These siRNAs are too short to trigger the nonspecific dsRNA responses, but they still promote degradation of complementary RNA sequences (21–23). We have directly compared the activity of optimized oligonucleotides that work by RNase H mechanism with those that work by an RNAi mechanism in human cells (24). The potency, maximal efficacy, duration of action, selectivity and efficiency for identification of leads was similar for both mechanisms in cell-based assays. The one noted difference between the 2 mechanisms, is that RNase H oligonucleotides are able to cleave pre-mRNA in the nucleus, whereas siRNA oligonucleotides appear to only be able to interact with mature mRNA in the cytoplasm. These results suggest that both mechanisms are equally valid for inhibition of gene expression in mammalian cells.

There are other RNases present in cells that may be exploited in a manner similar to RNase H or the dsRNase associated with RNAi. As an example, Wu et al., reported that single-stranded, phosphorothioate-modified oligoribonucleotides can promote selective loss of ha-ras mRNA in human cells (25). The RNA oligonucleotides could be partially modified with 2′-O-methyl nucleosides and still support enzyme activity. The enzyme activity is consistent with a RNase III type enzyme. RNase III activity is present in both cytosolic and nuclear extracts (25). It is unclear if this enzyme activity is the same RNase III used for siRNA oligonucleotides. Recent work has demonstrated that single-stranded RNA oligonucleotides can interact with the RISC and promote selective degradation of targeted RNA, consistent with RNAi activity, albeit not as efficiently as double-stranded RNA (26,27). Another RNase enzyme that has been exploited for antisense applications is RNase L (28). RNase L is ribonuclease activated by 2′-5′-linked oligoadenylates generated in response to interferon activation. Selectively, linkage of 2′-5′ oligoadenylate to an antisense oligonucleotide has been reported to promote selective cleavage of the targeted mRNA (28–30).

It should be noted that not all oligonucleotide designed to hybridize to a target RNA effectively inhibit target gene expression (2,31–33). This is believed to be due to inaccessibility of some regions of the RNA to the oligonucleotide due to secondary or tertiary structure or to protein interactions with the RNA. At this time, there are no good predictive algorithms for predicting antisense oligonucleotide-binding sites

Table 1 Antisense Oligonucleotides Approved or Currently in Clinical Development

Oligonucleotide	Molecular target	Disease indication	Chemistry	Route of administration	Status	Sponsor
Vitravene (fomivirsen, ISIS 2922)	Human CMV IE-2 gene	CMV retinitis	Phosphorothioate oligodeoxynucleotide	Intravitreal	Marketed	Novartis Ophthalmic/ISIS Pharmaceuticals
LY900003 (Affinitak, ISIS 3521)	Protein kinase C-α	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase III/II	Lilly/ISIS
Oblimersen (Genasense, G3139)	BCL-2	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase III/II	Aventis/Genta
Alicaforsen (ISIS 2302)	ICAM-1	Crohn's disease, ulcerative colitis	Phosphorothioate oligodeoxynucleotide	Intravenous/ enema	Phase III/II	ISIS Pharmaceuticals
ISIS 2503	ha-*ras*	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	ISIS Pharmaceuticals
EPI-2010	Adenosine A1 receptor	Asthma	Phosphorothioate oligodeoxynucleotide	Inhaled	Phase II	EpiGenesis
ISIS 14803	HCV RNA	HCV	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	ISIS Pharmaceuticals
GTI-2040	Ribonucleotide reductase R1 subunit	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	Lorus Therapeutics
GTI-2501	Ribonucleotide reductase R2 subunit	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	Lorus Therapeutics
LErafAON	c-raf kinase	Cancer-radiosensitizer	Liposome formulation of phosphorothioate oligodeoxynucleotide	Intravenous	Phase I/II	NeoPharm
AP12009	TGF-B2	Malignant glioma	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase I/II	Antisense Pharma
Gem-231	Protein kinase A	Cancer	Phosphorothioate 2′-*O*-methyl/ oligodeoxynucleotide chimera	Intravenous	Phase II	Hybridon
MG98	DNA Methyltransferase	Cancer	Phosphorothioate 2′-*O*-methyl/ oligodeoxynucleotide chimera	Intravenous	Phase II	MethyGene/MGI Pharma/British Biotech
ISIS 104838	TNF-α	Rheumatoid arthritis	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Subcutaneous/ Oral	Phase II	ISIS Pharmaceuticals
OGX-011 (ISIS 112989)	Clusterin	Cancer	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Intravenous/ subcutaneous	Phase I/II	Oncogenix/ISIS Pharmaceuticals
ISIS 113715	PTP-1B	Diabetes	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Subcutaneous	Phase I	ISIS Pharmaceuticals
ATL1102 (ISIS 107248)	CD49D (alpha subunit of VLA4)	Multiple sclerosis	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Subcutaneous	Phase I	Antisense Therapeutics Ltd/ISIS Pharmaceuticals
Resten-NG	c-myc	Restenosis	Morpholino	Catheter delivery-intra-arterial	Phase II	AVI BioPharma
Oncomyc-NG	c-myc	Cancer	Morpholino	Unknown	Phase I/II	AVI BioPharma
AVI-4126	c-myc	Polycystic kidney disease	Morpholino	Intravenous	Phase I	AVI BioPharma
AVI-4557	Cytochrome P450 (CYP3A4)	Inhibit drug metabolism	Morpholino	Intravenous	Phase I	AVI BioPharma

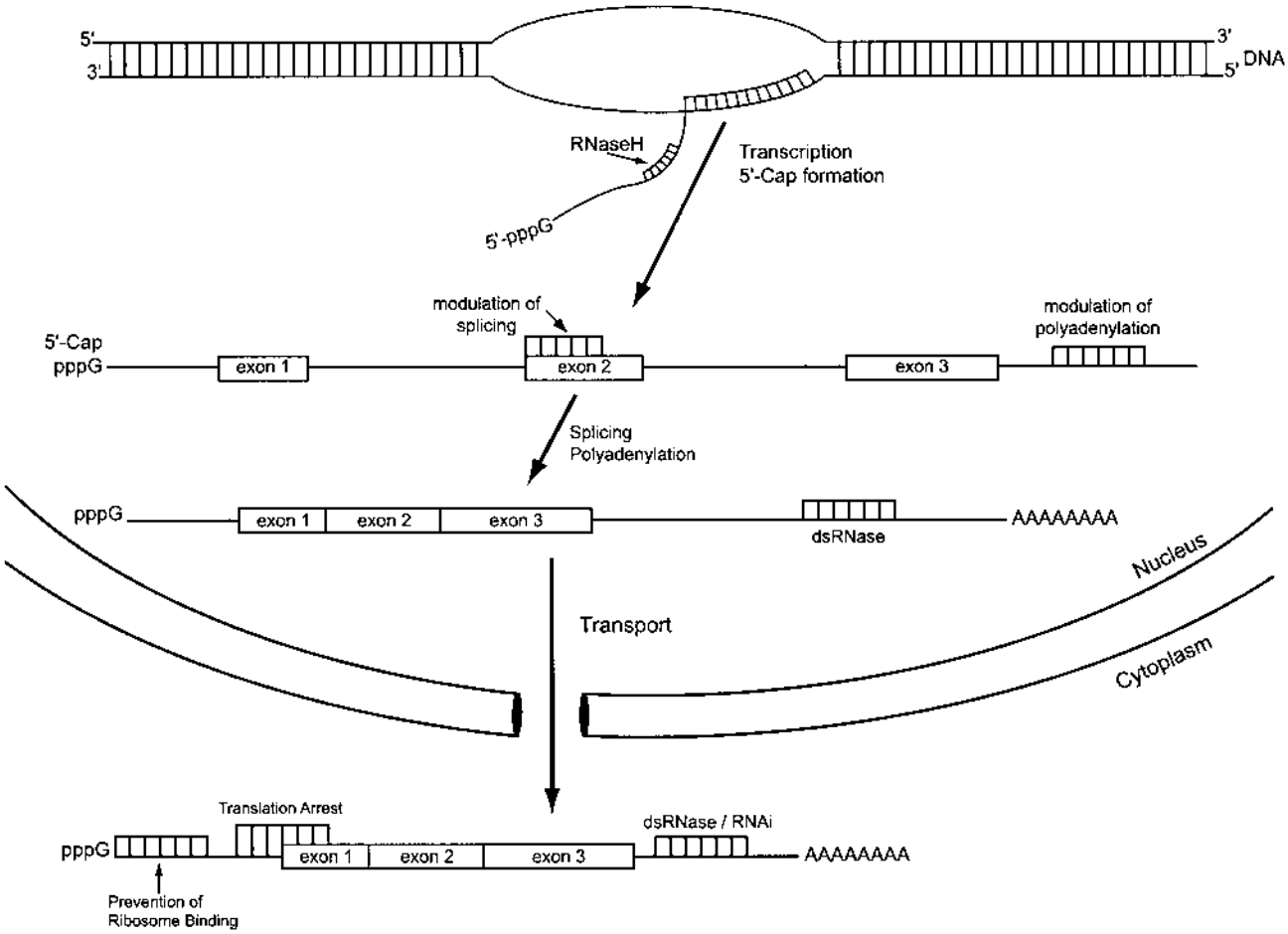

Figure 2 Antisense mechanisms of action. Cartoon depicting 3 different mechanisms by which an antisense oligonucleotide can inhibit expression of a targeted gene product by hybridization to the mRNA, or pre-mRNA which codes for the gene product.

on a target RNA. In our experience, we have found active oligonucleotides that work through an RNase H-dependent mechanism can hybridize to any region on the mRNA or pre-mRNA. Thus, some serendipity is still involved in the process of identifying and optimizing potent and effective antisense inhibitors.

Early on it was believed that occupancy of the RNA (the receptor for the antisense oligonucleotide) by the oligonucleotide would be sufficient to block translation of the RNA (i.e., translation arrest) (34). Subsequent studies have documented that oligonucleotides are not efficient at blocking translation of mRNA if they bind 3′ to the AUG translation initiation codon. Furthermore, we have found that only certain sites in the 5′-untranslated region of a mRNA are effective target sites for an antisense oligonucleotide. In particular, the 5′-terminus of a transcript appears to be a good target site for oligonucleotides for some molecular targets in that occupancy of this region prevents assembly of the ribosome on the RNA (35). It should be noted that occupancy of the receptor (RNA) and steric blocking of factor binding by high-affinity oligonucleotides can be an efficient mechanism for blocking gene expression. For the example cited above, the steric blocking oligonucleotide was approximately 10-fold more potent than an oligonucleotide that supports RNase H activity. These results suggest that catalytic turnover of the target RNA is not the rate-limiting step for antisense oligonucleotides.

Another process that noncatalytic oligonucleotides can use to alter gene expression is through regulating RNA processing. Most mammalian RNAs undergo multiple post- or cotranscriptional processing steps, including addition of a 5′-cap structure, splicing, and polyadenylation. Because single-stranded antisense oligonucleotides localize to the cell nucleus (36–39), they have the potential of regulating these processes.

Several studies have been published documenting that antisense oligonucleotides can be used to regulate RNA splicing in both cell-based assays and in rodent tissues (40–47). Oligonucleotides can be used to modulate alternative splicing by promoting use of cryptic splice sites as was exemplified for β thalassemia (40,41), or by enhancing use of an alternative splice site. Oligonucleotide binding to the pre-mRNA can also be exploited to mask polyadenylation signals on the pre-mRNA, forcing the cell to use alternative poly A sites (48). Finally, oligonucleotides, in principle, can regulate RNA function by sterically preventing factors from binding or changing the structure of the RNA such that it is no longer recognized by the factor. Thus, there are multiple mechanisms by which oligonucleotides can be use to inhibit or modulate expression of a target gene product. No single mechanism is far superior to other mechanisms, thus one should tailor the mechanism for the specific biological application.

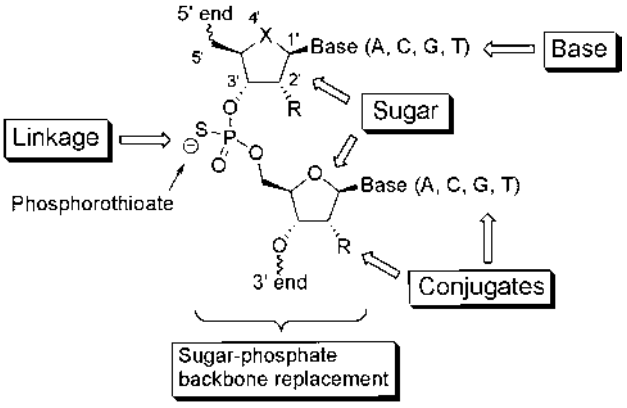

Figure 3 Positions that have been chemically modified for antisense oligonucleotides.

III. ANTISENSE OLIGONUCLEOTIDE CHEMISTRY

The most advanced oligonucleotide chemistry used for antisense drugs is phosphorothioate oligodeoxynucleotides. These differ from natural DNA in that 1 of the nonbridging oxygen atoms in phosphodiester linkage is substituted with sulfur (Fig. 1). Phosphorothioate oligodeoxynucleotides are commercially available, easily synthesized, support RNase H activity, exhibit acceptable pharmacokinetics for systemic and local delivery, and have not exhibited major toxicities that would prevent their use in humans. There have been significant resources employed to identify chemical modifications that further improve upon the properties of phosphorothioate oligodeoxynucleotides. The primary objectives of the effort are similar to medicinal chemistry efforts for other types of pharmacological agents (i.e., to increase potency, improve pharmacokinetics, and decrease toxicity).

A dimer of an oligonucleotide depicting subunits that may be modified to enhance oligonucleotide drug properties is depicted in Fig. 3. In naturally occurring nucleic acids, these subunits are composed of heterocycles, carbohydrate sugars, and phosphodiester-based linkages between the sugars. The combination of the carbohydrate sugar (ribose in RNA, 2′-deoxyribose in DNA) and the linkage provides the backbone of the oligonucleotide polymer. Many modifications have been made on the individual base, sugar, and linkage subunits, and the sugar-phosphate backbone has been completely replaced with an appropriate substitute. In Addition, many diverse moieties have been conjugated to various positions in the subunits, mainly in an attempt to alter the biophysical properties of the polymer. Finally, prodrug modifications may be employed to enhance drug properties. Most of the positions available in a nucleoside dimer (approximately 25 positions for each dimer that do not directly interfere with Watson–Crick base pair–hydrogen bonding) have been modified and studied for their effects on the properties of the resulting oligonucleotides.

The nucleobases or heterocycles of nucleic acids provide the recognition points for the Watson–Crick base pairing rules and any oligonucleotide modification must maintain these specific hydrogen-bonding interactions. Thus, the scope of heterocyclic modifications is somewhat limited. The relevant heterocyclic modifications can be grouped into 2 structural classes: (1) those that enhance base stacking, and (2) those that provide additional hydrogen bonding. The primary objective of heterocyclic modifications being to enhance hybridization, resulting in increased affinity (Fig. 4). Modifications that enhance base stacking by expanding the π-electron cloud are represented by lipophilic modifications in the 5-position of pyrimidines, such as propynes, hexynes, azoles, and a simple methyl group (49–52) and the 7 position of 7-deaza-purines position, including iodo, propynyl, and cyano groups (53–55). Investigators have continued to build out of the 5-position of cytosine by going from the propynes to 5-membered heterocycles to tricyclic fused systems emanating from the 4 and 5-positions of (Fig. 4) (56–59). A second type of heterocycle modification is represented by the 2-aminoadenine (Fig. 4), where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the 3 hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified A (55) and the G-clamp, a tricyclic cytosine analog having hydrogen-bonding capabilities in the major groove of heteroduplexes (58) (Fig. 4). Furthermore, N2-modified 2-amino purine oligonucleotides have exhibited interesting binding properties (60,61). All these modification are positioned to lie in the major or minor groove of the heteroduplex, do not affect sugar conformation of the heteroduplex, and provide little nuclease resistances, but will generally support an RNase H cleavage mechanism.

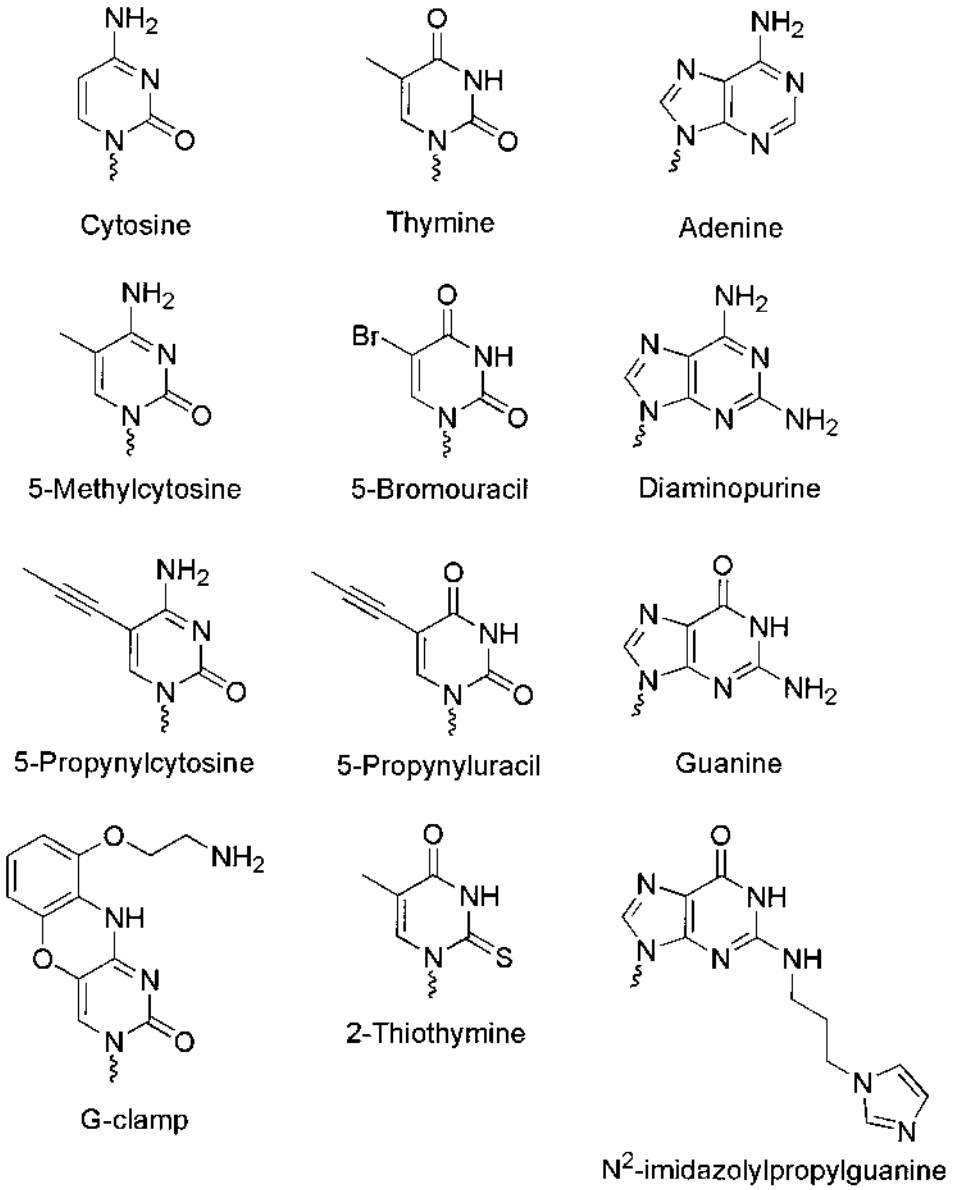

Figure 4 Examples of different heterocycle modifications that support antisense activity.

Modifications in the ribofuranosyl moiety have provided the most value in the quest to enhance oligonucleotide drug properties (Fig. 5). In particular, certain 2′-*O*- modifications have greatly increased binding affinity and nuclease resistance, altered pharmacokinetics, and are potentially less toxic (62). Preorganization of the sugar into a 3′-*endo* pucker conformation is responsible for the increased binding affinity (63–65). The 2′-*O*-methoxyethoxy (MOE) and 2′-*O*-methyl modifications (Fig. 5) are the most advanced of the 2′-modified series, and have entered clinical trials. The cationic 2′-*O*-aminopropyl (66) and 2′-*O*-(dimethylaminooxyethyl) (67,68) have shown favorable binding affinity, with dramatically improved nuclease resistance. In an attempt to extend on the increased nuclease resistance of these cationic modifications to the high affinity seen with MOE, a dimethylaminoethyl version (DMAEOE) was prepared. This modification displays hybridization properties equal to or superior to those of MOE, and nuclease resistance equal to that of the 2′-*O*-aminopropyl modification. The modification showing the largest known improvement in binding affinity is a bicyclic

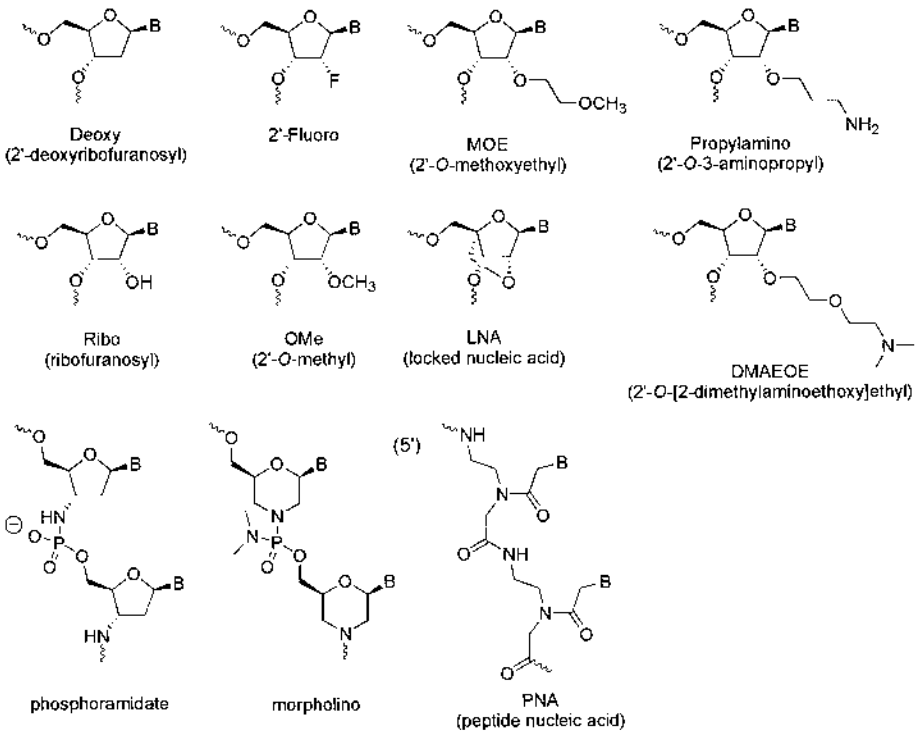

Figure 5 Examples of different sugar and backbone modifications that support antisense activity.

system having the 4′-carbon tethered to the 2′-hydroxyl group. As this modification ''locks'' the conformation of the ribose sugar into an RNA-like (3′-*endo*) conformation, it is referred to as locked nucleic acid (LNA) (69,70). LNA shows dramatically improved hybridization properties with regard to a reference DNA:RNA duplex, and has extremely high nuclease resistance. Although extremely promising from early biophysical and in vitro data, whether these properties will translate into improved efficacy in vivo remains to be seen.

It is now well known that uniformly 2′-*O*-modified oligonucleotides do not support an RNase H mechanism (71). The heteroduplex formed has been shown to present a structural conformation that is recognized by the enzyme, but cleavage is not supported (72–74). Thus, uniformly modified, ''RNA–like'' oligonucleotides (3′-*endo* sugar conformation) will be unable to effect cleavage of the target mRNA, and must therefore exert their effects via other means. This has led to the development of a chimeric strategy (3,71,75–77), which focuses on the design of high-affinity, nuclease-resistant antisense oligonucleotides that contain a ''gap'' of contiguous phosphorothioate- modified oligodeoxynucleotides (Fig. 6). On hybridization to target RNA, a heteroduplex is presented that supports an RNase H-mediated cleavage of the RNA strand via interaction with the 2′-deoxy gap region. The stretch of the modified oligonucleotide–RNA heteroduplex, which is recognized by RNase H may be placed anywhere within the modified oligonucleotide. The modifications in the flanking regions of the gap should not only provide nuclease resistance to exo- and endonucleases, but also not compromise binding affinity and base pair specificity. There are several types of structures that have been successfully developed (Fig. 6), with the most advance being ''gapmers,'' having a 7- to 10-base oligodeoxynucleotide gap flanked by 2 regions of 2′-modified nucleosides. These oligomers, in particular, 2′-MOE modified, show reduced toxicity, increased potency, and superior pharmacokinetics relative to the parent unmodified 20-mer phosphorothioate oligodeoxynucleotide (77–81).

Several possible mechanisms exist for uniformly modified, non-RNase H activating oligonucleotides to show efficacy, such as prevention of assembly of the ribosome through binding in the 5′-UTR, ''translation arrest,'' or ribosome stalling by blocking the reading of the mRNA ribosome, and modulation of splicing events by binding to splice junctions. Although all these strategies have been pursued, no uniformly modified oligonucleotides have advanced beyond gapmer oligonucleotides. However, much recent progress has been made with non-RNase H active oligonucleotides, and there remains much potential for these modifications. LNA and MOE have been used in a uniform context in addition to the gapmer strategy, and early studies show promise. Another interesting uniform

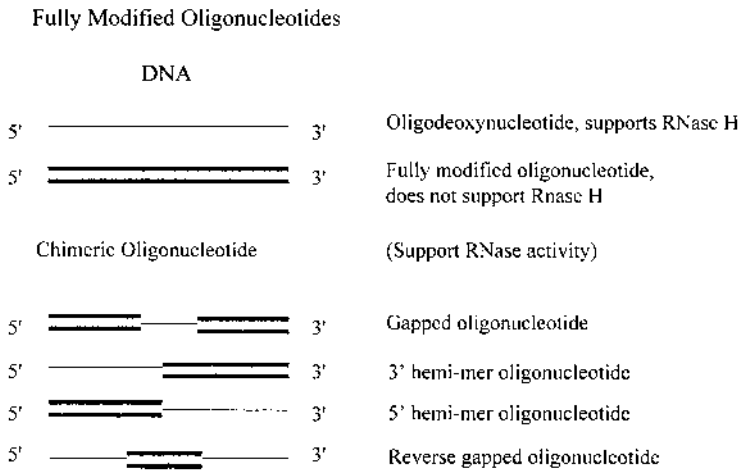

Figure 6 Examples of different oligonucleotide structures.

modification is the phosphoramidiate modification, which substitutes an amino group for the 3′ oxygen atom of the deoxyribose sugar of DNA. This results in a preference for the RNA-like (3′ endo) sugar conformation, and results in increased affinity as is seen with the 2′-*O*-alkyl modifications (82,83).

One of the most intriguing backbone oligonucleotide modifications is peptide nucleic acid (PNA). PNA is unique in that the sugar-phosphate backbone is completely replaced with a peptide-based backbone (Fig. 5) (84). This results in a polymer with a neutral backbone that has high affinity for complimentary nucleic acids. PNA has been extensively investigated as an antisense agent, but these efforts have generally been frustrated by the poor cellular penetration and in vivo pharmacokinetic properties of PNA (85). Recently, a 4-lysine peptide conjugated to a PNA was found to provide robust in vivo activity when targeted to a splice junction (47). These data are highly encouraging because they may provide a path to realizing the promise of PNA as an antisense therapeutic agent.

The most advanced uniform modification is the ''morpholino'' modification (Fig. 5), which is currently in phase II clinical trials for restenosis, cancer, and polycystic kidney disease. The morpholino modification simultaneously replaces the ribofuranosyl sugar with a morpholine ring, and the negatively charged phosphate ester with a neutral phosphorodiamidate linkage (86,87). Morpholinos are generally used around the translation initiation start codon, and are believed to function via translation arrest. A morpholino oligonucleotide has shown in vivo activity (88), as well as oral bioavailability in rats (89), which would be a major advance if studies proved general and translated to larger mammalian species.

In addition to heterocycle, backbone, and sugar modification discussed above, various pendant groups have been attached to oligonucleotides, such as cholesterol, folic acid, fatty acids, etc., to alter pharmacokinetic properties (90,91). The reader is referred to several recent reviews that discuss the chemistry of oligonucleotides in more detail (92–95). It should be noted that there is no single modification that covers all the desired properties for a modified oligonucleotide. Modifications have been identified that increase hybridization affinity of the oligonucleotide for its target RNA, increase nuclease resistance, decrease toxicity, and alter the pharmacokinetics (Table 2). Furthermore, the ideal oligonucleotide will differ for different applications. Therefore, it is important to be able to mix and match the various modifications to obtain the optimal oligonucleotide for the task at hand.

Table 2 Attributes of Various Modified Oligonucleotides

Attribute	Examples
Increased affinity for RNA	2′-*O*-methyl, 2′-fluoro, MOE, DMAEOE, LNA, 5-MeC, 5-propynyl, phenoxazine G-clamp, PNA, phosphoramidate, others
Increased nuclease resistance	MOE, DMAEOE, LNA, PNA, phosphoramidate, morpholino, others
Alter tissue distribution	MOE, PNA, cholesterol conjugate, phosphoramidate, morpholino, others
Decrease toxicity	2′-*O*-methyl, MOE, 5-MeC, morpholino, others

IV. PHARMACOKINETICS OF OLIGONUCLEOTIDES

A. Cellular Pharmacokinetics

Cellular uptake of phosphorothioate oligonucleotides has been documented to occur in most mammalian cells (96–103). Cellular uptake of oligonucleotide is time and temperature dependent. It is also influenced by cell type, cell culture conditions and media, and the length/sequence of the oligonucleotide itself (96). No obvious correlation between the lineage of cell, whether the cells are transformed or virally infected, and uptake has been identified. Cellular uptake appears to be an active process (i.e., oligonucleotide) will accumulate in greater concentration intracellular than in the medium and is energy dependent. Despite the fact that mammalian cells in culture will readily accumulate oligonucleotides, it has been necessary to further facilitate cytosolic delivery for many, but not all, cells with transfection agents such as cationic lipids, dendrimers, fusogenic peptides, electroporation, etc., (38,46,104–108). In the absence of these facilitators, it has been difficult to demonstrate true antisense effects in cultured cells., although there are some exceptions. However, in vivo, this is not the case. It has become apparent that in vitro cell uptake studies do not predict in vivo cell uptake and pharmacokinetics of oligonucleotides (96,109–113). Our understanding of cellular and subcellular uptake has evolved as superior analytical tools have been developed. These advances include development of immunohistochemical techniques use oligonucleotide-specific antibodies (114), and in situ perfusion of whole organs followed by cell sorting and subcellular separation techniques coupled with capillary gel electrophoresis (110).

Our understanding of cellular and subcellular distribution and pharmacokinetics of oligonucleotides in whole animals is emerging. In our laboratories, we use more specific tools for qualification and even quantification of intact oligonucleotide (110,114–116). Phosphorothioate oligonucleotides rapidly distribute to whole tissue with distribution half-lives range from 30 to 60 min in vivo. Approximately half of the oligonucleotide associated with the liver (as an example) is intracellular in both parenchymal and nonparenchymal cells by 4 h after intravenous administration (110,117). The other half of the organ-associated oligonucleotide appears to be associated with extracellular matrix or interstitium, or loosely bound to the cell membrane. Consistent with this observation, others have shown that phosphorothioates have been localized to connective tissue and can bind to various proteins within these matrices, such as laminin and fibronectin (114,118,119). Some of this matrix- associated oligonucleotide will diffuse to cells over time or be lost to efflux from the organ (114). It is likely that both of these processes are functioning up to 24 h after administration of oligonucleotide. By 24 h after injection of phosphorothioate oligonucleotide, little is seen to be associated with extracellular matrix (114). Thus, it is likely that whole organ pharmacokinetic evaluation after 24 h will parallel cellular clearance kinetics.

Although the in vitro studies fail to predict well which cell types will take up oligonucleotide in vivo, the general trend of variability from cell type to cell type continues to be observed in vivo (114). Based on these results, one would not expect to uniformly inhibit expression of a targeted gene product within a tissue or whole organism, resulting in differential sensitivity of different tissues and cells within tissue to the antisense effect. Subcellular distribution has been shown to be broad, and the extent of cytosolic and nuclear distribution differs between cells (110). In general, the total number of oligonucleotide molecules is greatest in the cytosol. However, because of the much smaller volume of the nucleus, the nucleus may often contain a higher concentration of oligonucleotide than the cytosol.

Nuclease metabolism has been shown to account for the clearance of phosphorothioate oligonucleotide from organs of distribution. Within the cells, the pattern of metabolites appears to be quite similar between cell types and the subcellular compartments (membrane associated, cytosolic, and nuclear). Increasing doses from 5 to 50 mg/kg only moderately decreased metabolism intracellularly, consistent with whole organ data (110).

Several studies have suggested that active uptake processes, including receptor-mediated endocytosis and pinocytosis, are involved in uptake of oligonucleotides in vivo. At very low doses (less than 1 mg/kg), competition of binding for scavenger receptors in vivo altered the whole organ distribution of oligonucleotides in liver but not in kidney (120–122). However, distribution studies conducted in scavenger receptor knockout mice did not show significantly altered intracellular and whole organ distribution of phosphorothioate oligonucleotides (123).

Distribution in the kidney has been more thoroughly studied, and drug has been shown to be present in Bowman's capsule, the proximal convoluted tubule, the brush border membrane, and the renal tubular epithelial cells (114,124). These data suggested that the oligonucleotides are filtered by the glomerulus and then reabsorbed by the proximal convoluted tubule epithelial cells. Moreover, the authors identified a specific protein in the brush border that may mediate uptake. In subsequent studies, the authors have purified the 45-kDa protein, reconstituted it in phospholipid vesicles and demonstrated that it served as a channel allowing nucleic acid to pass through phospholipid bilayers (125). In separate studies, other investigators have shown that, although some oligonucleotide is taken up from the tubular lumen brush border, the distribution to the tubule epithelial cells is predominantly from the capillary serosal side (126). The uptake from capillary circulation may not be receptor mediated. In summary, it is likely that there are multiple processes involved in the uptake of oligonucleotides into cells in vivo. Additional research will be required to further elucidate these mechanisms.

B. Whole Animal Oligonucleotide Pharmacokinetics

1. Phosphorothioate Oligodeoxynucleotides

The plasma pharmacokinetics of phosphorothioate oligodeoxynucleotides are characterized by rapid and dose-dependent

clearance (30–60 min half-life) driven primarily by distribution to tissue and secondarily by metabolism. Urinary and fecal excretion are minor pathways for elimination of phosphorothioate oligonucleotides. Dose-dependent clearance from plasma is predominantly a function of saturable tissue distribution (127,128). Metabolism has been shown to be unchanged in plasma over a large dose range (1–50 mg/kg) and after repeated administration up to 1 month, suggesting that metabolism is neither inhibited or induced by repeat administration (129).

The plasma pharmacokinetics are quite similar between animals and man, and they scale from one species to the next on the basis of body weight, not surface area (129–133). For example, it is possible to show that, when dosed on the basis of body weight, the concentrations of oligonucleotides in plasma administered by a 2-h constant intravenous infusion are similar between humans and monkeys. Thus, it has been possible to predict plasma concentrations in humans from nonclinical pharmacokinetic data.

Phosphorothioate oligonucleotides bind to circulating plasma proteins such as albumin and α-2 macroglobulin (134). The apparent affinity for human serum albumin is low (10–30 μM). Therefore, plasma protein binding provides a repository for these drugs preventing rapid renal excretion. Because serum protein binding is saturable at high concentrations, intact oligonucleotide may be found in urine in increasing amounts as dose rate and/or amount is increased (129, 135,136).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after intradermal, subcutaneous, intramuscular, or intraperitoneal administration (109,127,137,138). Nonparenteral absorption has been characterized for pulmonary and oral routes of administration. Estimates of bioavailability range from 3% to 20% following intranasal dosing and $< 1\%$ by the oral route (139,140). Although it is likely that permeability in the intestine is low, stability of these compounds in the intestine (prior to absorption) may be a rate-limiting factor to oral absorption (141,142). As discussed below, some chemical modifications to the oligonucleotide enhance oral absorption. The metabolic half-life of a 20-mer phosphorothioate oligonucleotide in the rat intestine (in vivo) is less than 1 h (data shown in Section VI).

Phosphorothioates are broadly distributed to all peripheral tissues. Highest concentrations of oligonucleotides are found in the liver, kidney, spleen, lymph nodes, and bone marrow with no measurable distribution to the brain (109,127,129, 135,141). Many other tissues take up smaller amounts of oligonucleotide, resulting in lower tissue concentrations. Phosphorothioate oligonucleotides are primarily cleared from tissues by nuclease metabolism. Rate of clearance differs between tissues with the spleen, lymph nodes, and liver, generally clearing more rapidly than kidney, for example. In general, the clearance rates result in half-lives of elimination ranging from 2 to 5 days in rodents and primates (128,133).

In summary, pharmacokinetic studies of phosphorothioate oligonucleotides demonstrate that they are well absorbed from parenteral sites, distribute broadly to all peripheral tissues, do not cross the blood–brain barrier, and are eliminated primarily by slow metabolism. In short, once-a-day or every-other-day systemic dosing should be feasible. In general, the pharmacokinetic properties of this class of compounds appear to be largely driven by chemistry rather than sequence. Additional studies are required to determine whether there are subtle sequence-specific effects on the pharmacokinetic profile of this class of drugs.

2. Second-generation Oligonucleotides

The plasma pharmacokinetics of 2′-*O*-methyl-, 2′-*O*-propyl-, or 2′-*O*-methoxyethyl-modified oligonucleotides do not differ significantly from their oligodeoxynucleotide congeners (79,80,143,144). Because metabolism plays only a minor role in the plasma distribution kinetics, this modification is expected to do little to alter the distribution and excretion kinetics. Early studies in our laboratory indicate that the binding affinity to serum albumin may be somewhat lessened by 2′-ribose sugar modifications, but the overall capacity of the plasma proteins to bind these oligonucleotides is not significantly changed (Table 3). Therefore, urinary excretion remains a minor route of elimination, and these compounds are broadly distributed to peripheral tissues.

Several of the 2′-ribose sugar modification produces enough of an increase in nuclease resistance that it is possible

Table 3 Serum Albumin Affinity, Whole Plasma Fraction Bound to Proteins (F_b), and Fraction of Dose Excreted in Urine ($f_{excreted}$, 0–24 h) Following Intravenous Administration at 3 mg/kg—Comparison of First- and Second-Generation Chemistries

Compound no.	Chemistry	Kd (μM)	$F_{b\,(\%)}$	$f_{excreted}$
ISIS 2302	PS ODN[a]	17.7	99.2	0.003
ISIS 11159	PS 2′-MOE[b]	29.3	95.5	0.032
ISIS 16952	PO 2′-MOE[c]	>500	79.6	0.45

[a]PS ODN, phosphorothioate oligodeoxynucleotide.
[b]PS 2′-MOE, 2′-O-methoxyethyl ribose modified phosphorothioate (all nucleotides were modified).
[c]PO 2′-MOE, 2′-O-methoxyethyl ribose modified phosphodiester (all nucleotides were modified).

to produce relatively stable oligonucleotides with phosphodiester linkages (Table 2). Thus, this modification allows for elimination or reduction in the number of sulfurs contained in the internucleotide bridge, but these compounds are less stable than their 2′-modified phosphorothioate congeners (145). In addition, as sulfur is removed, plasma protein binding is greatly decreased and rapid removal from plasma by filtration in the kidney increases significantly. This pharmacokinetic characteristic may limit the use of phosphodiester second-generation modified oligonucleotides intended for treatment of systemic disease (79). Alternatively, this pharmacokinetic profile may be ideal for locally administered oligonucleotides because it limits the accumulation of systemically absorbed drug.

Absorption for parenterally administered modified oligonucleotides is consistently rapid and nearly complete. Some of the second-generation modified oligonucleotides have exhibited improved intestinal permeability (141) as well as significantly improved stability in the intestine (142). It is likely this combination of improved biochemical characteristics have led to the observation of improved oral bioavailability (141) for this class of oligonucleotide compounds.

The distribution pattern of the 2′-ribose-modified phosphorothioate oligonucleotides are similar to first-generation phosphorothioates and similarly not altered by changes in sequence. Kidney, liver, spleen, bone marrow, and lymph nodes are the major sites of distribution. The most exciting difference in pharmacokinetics is, not surprisingly, manifested in prolonged terminal elimination half-lives from tissues of distribution. The elimination half-lives appear to be increased nearly 5 to 10-fold, suggesting that once-weekly systemic dosing may be feasible (Table 4).

In summary, pharmacokinetic studies of 2′-modified ribose phosphorothioate oligonucleotides demonstrate that they are well absorbed from parenteral sites, may have improved oral absorption attributes, and distribute broadly to all peripheral tissues. Although stability has been greatly enhanced, nuclease metabolism is likely the primary mechanism for ultimate elimination of these modified oligonucleotides. In short, once-a-week systemic dosing should be feasible and oral administration may be possible in the near future. Additional studies are required to determine whether there are substantial sequence-specific effects on the pharmacokinetic profile of this class of drugs.

Table 4 Summary of Observed Organ Clearance Half-lives (in days) Comparing Second- and First-Generation Chemistries

Organ	2′-Modified phosphorothioate oligonucleotide	Phosphorothioate oligodeoxynucleotide
Kidney cortex	21.7	5.0
Kidney medulla	10.4	3.1
Liver	7.7	2.8
Spleen	8.1	3.3
Lymph nodes	16.5	0.9
Bone marrow	11.5	1.3

V. TOXICOLOGY OF OLIGONUCLEOTIDES

Phosphorothioate oligodeoxynucleotides have been examined extensively in a full range of acute, chronic, and reproductive studies in rodents, lagomorphs, and primates. At high doses, there is a distinctive pattern of toxicity that is common to all phosphorothioate oligodeoxynucleotides (146–149). The remarkable similarity in toxicity with different phosphorothioate oligodeoxynucleotides suggests that, for this class of antisense compounds, toxicity is independent of sequence and is the result of nonantisense-mediated mechanisms. The most probable mechanism of the observed toxicities is the binding of oligodeoxynucleotides to proteins. These nonantisense-mediated pathways are believed to be responsible for most, if not all, of the toxicities associated with the administration of these compounds to laboratory animals. This conclusion is strengthened by studies in which little or no differences in toxicity are observed between pharmacologically active and inactive sequences. Different patterns of toxicity exist between rodents and primates. Understanding the mechanisms behind these differences is crucial to understanding which species best predicts the potential human effects. A comparison of the toxicological profiles of phosphorothioate oligodeoxynucleotides with that of the next generation of phosphorothioate oligonucleotides suggests that some of the chemical class-related toxicities of phosphorothioate oligodeoxynucleotides can be ameliorated by chemical modification.

A number of phosphorothioate oligodeoxynucleotides have been examined in 1 or more of the following battery of genotoxicity assays: Ames test, in vitro chromosomal aberrations, in vitro mammalian mutation (HGPRT locus and mouse lymphoma), in vitro unscheduled DNA synthesis tests, and in vivo mouse micronucleus. In all these assays, the results were negative and there was no evidence of mutagenicity or clastogenicity of these compounds (150).

A. Acute Toxicities

In rodents, the acute toxicity of phosphorothioate oligodeoxynucleotides has been characterized as part of an effort to determine the maximum tolerated dose for in vivo genotoxicity assays. The doses of 3 phosphorothioate oligodeoxynucleotides required to produce 50% lethality (LD50) were estimated to be approximately 750 mg/kg (150).

In primates, the acute dose-limiting toxicities are a transient inhibition of the clotting cascade and the activation of the complement cascade (146,151,152). Both of these toxicities are believed to be related to the polyanionic nature of the molecules and the binding of these compounds to specific protein factors in plasma.

Prolongation of clotting times following administration of different phosphorothioate oligodeoxynucleotides is charac-

terized by a concentration-dependent prolongation of activated partial thromboplastin times (aPTT) (149,153–155). The prolongation of aPTT is highly transient and directly proportional to plasma concentrations of oligodeoxynucleotide and therefore parallels the plasma drug concentration curves with various dose regimens. As drug is cleared from plasma, the inhibition diminishes such that there is complete reversal within hours of dosing. With repeated administration, there is no evidence of residual inhibition. Prolongation of aPTT has been observed in all species examined to date, including human, monkey, and rat. The mechanism of prolongation of aPTT by phosphorothioate oligodeoxynucleotides is believed to be a result of the interaction of the oligonucleotides with proteins. It is well known that polyanions are inhibitors of clotting, and phosphorothioate oligodeoxynucleotides may act through similar mechanisms. If these oligonucleotides inhibit the clotting cascade as a result of their polyanionic properties, then binding and inhibition of thrombin would be a likely mechanism of action. However, the greater sensitivity of the intrinsic pathway to inhibition by phosphorothioate oligodeoxynucleotides suggests that there are other clotting factors specific to this pathway that may also be inhibited. Recent data suggest that there is a specific allosteric inhibition of the tenase complex as well as binding to thrombin (152,156).

In clinical trials with ISIS 2302, normal volunteers and patients were dosed with 2 mg/kg infused over 2 h. This regimen produced total oligonucleotide concentrations of 10 to 15 μg/mL and a concomitant increase in aPTT of approximately 50% (130), which correlates well with in vitro human and animal data. The transient and reversible nature of aPTT prolongation, combined with the relatively small magnitude of the change, makes these effects clinically insignificant for the current treatment doses and regimens.

Activation of the complement cascade by phosphorothioate oligodeoxynucleotides has the potential to produce the most profound acute toxicological effects. In primates, treatment with high doses over short infusion times resulted in marked hematological effects and marked hemodynamic changes that are believed to be secondary to complement activation. Hematological changes are characterized by transient reduction in neutrophil counts, presumably due to margination, followed by neutrophilia with abundant immature, nonsegmented neutrophils (147,151). In a small fraction of monkeys, complement activation was accompanied by marked reductions in heart rate, blood pressure, and subsequently cardiac output. In some animals, these hemodynamic changes were lethal (146,151,157).

There is an association between cardiovascular collapse and complement activation. That is, all monkeys demonstrating some degree of cardiovascular collapse or hemodynamic changes had markedly elevated levels of complement split products. However, the converse is not true, in that only a fraction of the animals with activated complement had cardiovascular functional changes (150). Thus, this observation suggests that there may be sensitive subpopulations or predisposing factors within individual animals that make them susceptible to the physiological sequelae of complement activation. Because of these observed hemodynamic changes, primate studies to monitor for these effects have become part of the normal evaluation of these compounds (158,159). Although complement activation at high doses is consistent and predictable between animals, there is currently little appreciation for the variability in the severity of the associated hemodynamic changes. Although the split product Bb can be used to monitor complement activation, it is C5a (complement split product) that is the most biologically active split product. Preliminary data obtained relating response to complement split product levels indicate that C5a levels are elevated more significantly in some of the more affected animals (150).

The goal of toxicity studies is to characterize the toxicity of compounds and to establish a framework upon which clinical safety studies can be designed. In this regard, it is useful to examine the relationship between plasma concentrations of oligonucleotides and the activation of complement. When Bb concentrations were plotted against the concurrent plasma concentrations of oligodeoxynucleotides in primates, it was apparent that complement was only activated at concentrations of phosphorothioate oligodeoxynucleotides that exceed a threshold value of 40 to 50 μg/mL (151). Bb levels remained unchanged from control values at plasma concentrations below the threshold. Remarkably, this threshold concentration is similar for three 20-mer phosphorothioate oligodeoxynucleotides and for an 8-mer phosphorothioate oligodeoxynucleotide that forms a tetrad complex (160,161). Recent data demonstrate that human serum may be less sensitive to activation than monkey serum, suggesting a species difference in sensitivity. Regardless of small differences, it is clear that clinical dose regimens should be designed to avoid plasma oligodeoxynucleotide concentrations that exceed 40 to 50 μg/mL. To this end, the similarities in plasma pharmacokinetics between monkeys and humans have allowed the design of dose regimens that achieve desired plasma concentration profiles.

The most direct approach for staying below the plasma thresholds for complement activation is to reduce the dose rate by substituting prolonged infusions for bolus injections. In clinical trials with phosphorothioate oligodeoxynucleotides, the drugs are administered either as 2-h infusions or as constant 24-h infusions. At a rate of infusion of 2 mg/kg over 2 h, the Cmax was 8 to 15 μg/mL, still well below the threshold for complement activation (130). Phosphorothioate oligodeoxynucleotides have been administered by intravenous infusion to more than 3000 patients and volunteers without any significant indication of activation of the alternative complement cascade.

1. Modified Oligonucleotides

Chemical modifications to phosphorothioate oligodeoxynucleotides may reduce the potential to activate complement. In one study, cynomolgus monkeys were administered an intravenous infusion over a 10-min period with a 5, 20, or 50 mg/kg dose of a 17-mer phosphodiester oligodeoxynucleotide, Ar177, that had phosphorothioate caps on the 3′ and 5′ termini (154,162). This oligonucleotide is known to have a complex secondary structure. In this experiment, although there was

a dose-related increase in plasma concentrations of Bb, the magnitude of the increases were small in comparison to the known activity of full-phosphorothioate oligodeoxynucleotides (162). Whether this diminished potential to activate the complement cascade is related to the reduction of phosphorothioate linkages or whether it is due to the complex secondary structure of this particular oligodeoxynucleotide was not established by these experiments. Some insight into this question was obtained in a second series of experiments performed with oligonucleotides that contained 2′-*O*-methoxyethyl modifications of the ribose sugar in 12 of the 20 nucleotides (149,150). Cynomolgus monkeys were treated by 10-min intravenous infusion with single doses of 1, 5, or 20 mg/kg of this 20-mer oligonucleotide that was either fully modified phosphorothioate linkages (ISIS 13650) or had phosphodiester wings and a central region of phosphorothioate linkages (9 linkages, ISIS 12854). The termini of both compounds contained six 2′-modified nucleotides. A third unmodified phosphorothioate oligodeoxynucleotide, ISIS 1082, was included as a positive control. The unmodified compound produced marked increases in Bb and severe cardiovascular effect at the dose of 5 mg/kg (30- to 60-fold over baseline). At 5 mg/kg, the aPTT values were 41 and 33 sec for the fully phosphorothioate and partially phosphorothioate 2′-modified oligonucleotides, respectively. In contrast, the unmodified phosphorothioate oligodeoxynucleotide produced an aPTT of 72 sec at the same dose. These data suggest that reduction in the number of phosphorothioate linkages reduced the inhibitory effects on aPTT and the activation of the complement cascade. However, the more important difference was that both 2′-*O*-methoxyethyl compounds were markedly less potent in activating complement than an unmodified oligodeoxynucleotide (D.K. Monteith, P.L. Nicklin, and A.A. Levin, unpublished observations, 1997). Although the safety profile of phosphorothioate oligodeoxynucleotides has proven satisfactory, the acute safety profile of the next generation of oligonucleotides may be improved by modification of the 2′-position of the ribose sugar with an alkoxy such as 2′-*O*-methyl or 2′-*O*-methoxyethyl and by reductions in phosphorothioate linkages.

B. Toxicological Effects Associated with Chronic Exposure

One of the characteristic toxicities observed with repeated exposure of rodents to phosphorothioate oligodeoxynucleotides is a profile of effects that can be described as immune stimulation. The profile is characterized by splenomegaly, lymphoid hyperplasia, and diffuse multiorgan mixed mononuclear cell infiltrates (149). The severity of these changes is dose dependent and most notable at doses equal to or exceeding 10 mg/kg. The mixed mononuclear cell infiltrates consisted of monocytes, lymphocytes, and fibroblasts and were particularly notable in liver, kidney, heart, lung, thymus, pancreas, and periadrenal tissues (148,163–165).

Although immune stimulation in rodents is believed to be a class effect of phosphorothioate oligodeoxynucleotides and not dependent on hybridization, sequence is an important factor in determining immunostimulatory potential (166–169). Immunostimulatory motifs have been described in the literature and involve palindromic sequences and CpG (cytosine-guanosine) motifs (169).

Among the most remarkable features of oligodeoxynucleotide-induced immune stimulation are the species differences. Rodents are highly susceptible to this generalized immune stimulation, whereas primates appear to be relatively insensitive to the effect at equivalent doses. Even 6 months of treatment of cynomolgus monkeys with 10 mg/kg of a 20-mer oligodeoxynucleotide, ISIS 2302, given every other day produced only a relatively mild increase in B cell numbers in spleen and lymph nodes of the primates with no change in organ weights. The mixed mononuclear cellular infiltrates in liver and other organs that are so characteristic of the response in rodents are absent even after long-term exposure in monkeys (149). It is known that rodents are more susceptible to the stimulatory effects of lipopolysaccharides, and much of the immune stimulation produced by oligodeoxynucleotides shares characteristics with lipopolysaccharide stimulation. Assuming results obtained in monkeys can be used to predict stimulation in humans, then the immunostimulatory effects may not be a prominent adverse effect in humans.

It is evident that there are both species and sequence differences involved in immune stimulation and that specific sequences should, if possible, be excluded from oligodeoxynucleotides. In long-term toxicity studies in rodents, the constant cell proliferation associated with immune stimulation may have promoter-like effects and may thus complicate the interpretation of rodent carcinogenicity studies. At this time, there are no reports of toxicity studies longer than 6 months, and the long-term sequelae of immune stimulation in rodents are at present merely speculation. More important, immune stimulation following systemic administration of phosphorothioate oligodeoxynucleotides does not appear to be clinically relevant.

Morphologic changes in the bone marrow of mice were observed after 2 weeks of treatment (3 doses/week) with 100 to 150 mg/kg phosphorothioate oligodeoxynucleotide There was reduction in number of megakaryocytes that was accompanied by a reduction of approximately 50% in circulating platelet counts (164). Reductions in platelets have been observed in rats treated with 21.7 mg/kg ISIS 2105 given every other day (148), but were not observed in primates administered 10 mg/kg. Similarly, a reduction in platelets was observed in mice, but not in monkeys treated for 4 weeks with ISIS 2302 at doses of 100 and 50 mg/kg every other day, respectively. Similar observations were made for ISIS 5132 with reductions in platelets at 20 and 100 mg/kg in mice and no observed effect in monkeys up to 10 mg/kg (163). These data suggest that the mouse may be more sensitive to these subchronic effects on platelets than nonhuman primates. However, in acute studies in primates, transient reductions in platelets are occasionally observed. These transient reductions in platelets occur acutely during 2-h infusions at doses of 10 mg/kg, reverse after completion of the infusion, and have not been associated with any measurable change in platelet number 24

to 48 h after subchronic or chronic treatment regimens (150). Thrombocytopenia has been reported in AIDS patients treated with GEM 91, a 27-mer phosphorothioate oligodeoxynucleotide (170).

Tissue distribution studies have shown that the liver and kidney are major sites of deposition of phosphorothioate oligodeoxynucleotide. In toxicity studies with phosphorothioate oligodeoxynucleotides, a variety of hepatic changes have been observed. The immune-mediated cellular infiltrates in rodent livers were discussed above. With high-dose administration of oligodeoxynucleotides in all species examined, there was a hypertrophic change in Kupffer cells accompanied by inclusions of basophilic material that was observed with hematoxylin and eosin staining. These basophilic granules have been identified as inclusions of oligodeoxynucleotide (114). Furthermore, it was demonstrated that the presence of these inclusions was related to dose.

Hepatocellular changes were not a prominent feature of toxicity in primates. In cynomolgus monkeys, 50 mg ISIS 2302 per kg administered every other day for 4 weeks by intravenous injection produced no morphologic indication of liver toxicity, although there was a slight (1.5-fold) increase in AST in this group (171). Following subcutaneous doses of ISIS 3521 and ISIS 5132 of up to 80 mg/kg every other day for 4 doses, there was Kupffer cell hypertrophy and periportal cell vacuolation, but no indication of necrosis and only a very slight increase in ALT (150). After 4 weeks of alternate-day dosing with 10 mg/kg via 2-h intravenous infusion of either ISIS 3521 or ISIS 5132, there were no alterations in AST or ALT, suggesting that at clinically relevant doses of these compounds, there was no evidence for hepatic pathology or tansaminemia. In clinical trials with ISIS 2302, ISIS 3521, and ISIS 5132 at doses of 2 mg/kg administered by 2-h infusion on alternate days for 3 to 4 weeks, there was no indication of hepatic dysfunction, nor was there any evidence of transaminemia.

Like Kupffer cells in the liver, renal proximal tubule epithelial cells take up oligodeoxynucleotide, as demonstrated by autoradiographic studies and immunohistochemistry as discussed previously (114,118,172,173) and by the use of special histologic stains (147). The appearance of basophilic inclusions is dose dependent in proximal tubule cells. Significant renal toxicity can be induced by extremely high doses. Doses of 80 mg/kg in rats and monkeys have induced both histologic and serum chemistry changes in the kidney (174). At clinically relevant doses, however, there was no indication of renal dysfunction. In 4-week or 6-month toxicity studies with phosphorothioate oligodeoxynucleotides, we observed a much more subtle type of morphologic change in the kidney. At a dose of 10 mg/kg on alternate days, here was a decrease in the height of the brush border and enlarged nuclei in some proximal tubule cells. These changes have been characterized as minimal to mild tubular atrophic and regenerative changes. At a dose of 3 mg/kg and below, these changes were only infrequently observed, if at all.

An important aspect of dose-dependent effects is characterization of exposure concentrations and their relationship to morphological changes. To assess exposure, concentrations of oligodeoxynucleotides have been measured in the renal cortex obtained in subchronic and chronic toxicity studies. Renal concentrations increase with increasing doses. The concentration of total oligodeoxynucleotide in the renal cortex associated with minimal to mild (although not clinically relevant) renal tubular atrophy or regenerative changes is approximately 1000 μg/g of tissue. The cortex concentrations of total oligodeoxynucleotide that are associated with moderate degenerative changes after subcutaneous doses of 40 to 80 mg/kg are greater than 2000 μg/g. At a clinically relevant dose of 3 mg/kg every other day, the steady-state concentration of total oligodeoxynucleotide in the kidney is in the range of 400 to 500 μg/g, thus demonstrating a significant margin of safety between the clinical doses and those doses associated with even the most minimal morphologic renal changes. Application of clearance and steady- state pharmacokinetic models suggests that continued administration of oligodeoxynucleotide at this dose should never achieve the renal concentrations associated with dysfunction (129). These models have been confirmed in 6-month chronic toxicology studies, where tissue concentrations measured at the end of 6 months of every-other-day dosing was no different than levels observed after 4 weeks of dosing at a similar or equivalent dose.

C. Chemical Modification of Oligodeoxynucleotides

Chemical modifications of oligodeoxynucleotides have been shown to reduce the potency of immune stimulation. The simplest modification with remarkable activity for reducing the immunostimulatory effects of oligodeoxynucleotides is the replacement of cytosine with 5-methyl cytosine. The methylation of a single cytosine residue in a CpG motif reduced [^{3}H]uridine incorporation and IgM secretion by mouse splenocytes. Methylation of a cytosine not in a CpG motif did not reduce the immunostimulatory potential (175). In our experience with mice, when sequences with 5-methyl cytosine are compared with the same sequence without methylation, the methylated sequence has a lower potency for inducing immune stimulation, as determined by spleen weights and immune cell activation (176,177).

Substitution of methylphosphonate linkages for phosphorothioate linkages on each of the 3′ and 5′ termini have also been reported to reduce the proliferative effects and the secretion of IgG and IgM compared 2 with the full phosphorothioate analog (178). This suggests that that this modification can also be used to ameliorate immune stimulation. The addition of 2′-*O*-methyl substituents also reduced immunostimulatory potential (178). The relative contribution of the uridine substitution and the 2′-methoxy substitution could not be differentiated in this experiment. The effect of 2′-alkoxy modifications on immunostimulatory potential needs further investigation. Finally, the effects of chemical modifications of phosphorothioate oligonucleotides on renal and hepatotoxicity are currently being investigated.

VI. OLIGONUCLEOTIDE FORMULATIONS

A. Physical-chemical Properties

Due to the presence of a mixture of diastereisomers, phosphorothioate oligodeoxynucleotides are amorphous solids possessing the expected physical properties of hygroscopicity, low-bulk density, electrostatic charge pick up, and poorly defined melting point prior to decomposition. Their good chemical stability allows storage in the form of a lyophilized powder, spray-dried powder or a concentrated, sterile solution; more than 3 years of storage is possible at refrigerated temperatures.

Due to their polyanionic nature, phosphorothioate oligodeoxynucleotides are readily soluble in neutral and basic conditions. Drug-product concentrations are limited (in select applications) only by an increase in solution viscosity. The counter ion composition, ionic strength, and pH also influence the apparent solubility. Phosphorothioate oligodeoxynucleotides have an apparent pKa in the vicinity of 2 and will come out of solution in acidic environments (i.e., the stomach). This precipitation is readily reversible with increasing pH or by acid-mediated hydrolysis.

Instability of phosphorothioate oligodeoxynucleotides have been primarily attributed to 2 degradation mechanisms: oxidation and acid-catalyzed hydrolysis. Oxidation of the (P = S) bond in the backbone has been observed at elevated temperatures and under intense ultraviolet light, leading to partial phosphodiesters (still pharmacologically active) and are readily monitored by anion-exchange high-performance liquid chromatography. Under acidic conditions, hydrolysis reactions followed by chain-shortening depurination reactions have been documented by length-sensitive electrophoretic techniques.

B. Parenteral Injections

Given the excellent solution stability and solubility possessed by phosphorothioate oligodeoxynucleotides, it has been relatively straightforward to formulate the first-generation drug products in support of early clinical trials. Simple, buffered solutions have been successfully used in clinical studies by intravenous, intradermal, and subcutaneous injections. Recently, the intravitreal route was approved for the first antisense drug application.

C. Topical Delivery for Diseases of the Skin

The barrier properties of human skin have been an area of multidisciplinary research for a long time. Skin is one of the most difficult biological membrane to penetrate, primarily due to the presence of stratum corneum (SC), which is composed of corneocytes laid in a brick-and-mortar arrangement with layers of lipid. The corneocytes are partially dehydrated, anuclear, metabolically active cells completely filled with bundles of keratin with a thick and insoluble envelope replacing the cell membrane (179). The primary lipids in the SC are ceramides, free sterols, free fatty acids, and triglycerides (180), which form lamellar lipid sheets between the corneocytes. These unique structural features of SC provide an excellent barrier to penetration of most molecules.

Therefore, as the primary barrier to transport of molecules to the skin, physical alteration in SC can result in improved skin penetration. Tape stripping and abrasion by repeated brushing reduced the SC barrier sufficiently to allow penetration of naked plasmid DNA and produced gene expression in skin at a level comparable to that after intradermal injection of naked plasmid DNA (181). Other studies have also shown an increase in oligonucleotide penetration upon physical removal of SC barrier (182–184).

1. Altering the Thermodynamic Properties of the Molecules

Increasing lipid partitioning to improve skin penetration has been evaluated using 2 techniques that alter the thermodynamic properties of oligonucleotide molecules. A complex of phosphorothioate oligonucleotide with hydrophobic cations such as benzalkonium chloride resulted in increased penetration through isolated hairless mouse skin that was explained on the basis of greater partitioning in lipid phase (184). Chemical modification of oligonucleotides to eliminate the negative charges also resulted in a size-dependent increase in the penetration of oligonucleotide into the skin when used with chemical penetration enhancers such as ethanol and dimethyl sulfoxide (183).

2. Electrical Field for Alteration of Skin Permeability

Iontophoresis, which involves application of electric field across the skin to induce electrochemical transport of charged molecules, is studied extensively for transdermal delivery of phosphorothioate oligonucleotides (185,186). The transdermal delivery was shown to be size dependent with steady-state flux values ranging from 2 to 26 pmol/cm^2 in isolated hairless mice skin. The steady-state flux also depended on the sequence, and not just the base composition, of the oligonucleotide. Molecular structure, therefore, is a key contributor to iontophoretically assisted transport of oligonucleotides (187–189). Electroporation a technique using much higher voltage than iontophoresis to cause formation of transient aqueous pathway in skin lipids, provides therapeutic levels (> 1 μM) of oligonucleotides in the viable tissues of the skin (190).

3. Formulations for the Alteration of Skin Permeability

Chemical penetration enhancers have recently been studied for increasing transdermal delivery of oligonucleotides or other polar macromolecules. Chemical-induced transdermal penetration results from a transient reduction in the resistance of the SC barrier properties. The reduction may be attributed to a variety of factors such as opening of intercellular junctions due to hydration (191), solubilization of SC lipids (192,193) or increased lipid bilayer fluidization (194). Types of chemicals known to be penetration enhancers include alkyl esters (195),

phospholipids (196), terpenes (197), nonionic surfactants (198), and laurocapram (Azone) (199). A combination of various surfactants and cosolvents can be used to achieve skin penetration with therapeutically relevant concentration of phosphorothioate oligonucleotides in the viable epidermis and dermis (200). The topical formulations produced significantly higher epidermal and dermal levels of oligonucleotide than those achieved by an intravenous injection at highest tolerated doses. This suggests that the topical route is more efficient in reaching all layers of the skin than systemic administration of phosphorothioate oligonucleotides.

Liposomes have been studied to transport oligonucleotides into the skin. They can increase the fluidity of skin lipid layers (similar to chemical enhancers) to facilitate transdermal permeation and can also carry encapsulated molecules through appendageal pathway (201,202). Mixture of a phosphorothioate oligodeoxynucleotide with a suspension of anionic or neutral lipids resulted in a slight increase in accumulation in epidermis and dermis (R. Mehta, unpublished, 1999). Using a combination of different delivery techniques and formulations, it appears to be feasible to deliver a therapeutically relevant amount of antisense oligonucleotide to the skin. In addition, preliminary results in our laboratory show a dose-dependent pharmacological effect consistent with the antisense mechanism of action of an ICAM-1 antisense oligonucleotide, ISIS 2302 (200). Studies are also underway to assess pharmacology and tissue kinetics of ISIS 2302 in human disease models.

D. Oral Delivery

Of the numerous barriers proposed by Nicklin and others (138) to the oral delivery of oligonucleotides, our experience has confirmed that 2 stand out as critical: instability in the gastrointestinal (GI) tract and low permeability across the intestinal mucosa. Given the formidable nature of these 2 barriers, it is not surprising that oral delivery of oligonucleotides has been considered impossible, or at best, difficult—as is the case with proteins, which has necessitated the latter's nonenteral administration in order to achieve systemic concentrations considered therapeutic. Nevertheless, progress has been made to address and/or understand each barrier with respect to oligonucleotides. (P = S)-oligonucleotides have a distinct advantage over proteins in that the former does not rely on secondary structure for activity. This provides freedom from concern over secondary structure destabilization and allows for (P = S)-oligonucleotide structural modifications to address both presystemic and systemic metabolism.

Natural DNA and RNA are rapidly digested by the ubiquitous nucleases found within the gut. As a consequence, oligonucleotides need to be stabilized in order to achieve a reasonable GI residence time to allow for absorption to occur. Surprisingly, phosphorothioate oligodeoxynucleotides were found to be rapidly degraded by nucleases found in the GI tract; therefore, additional protection from nuclease degradation is required to achieve significant oral bioavailability. Oligonucleotides that are uniformly modified or modified on the 3′-end (gapmers or 3′-hemimers) (Fig. 6) with nuclease-resistant modification have the potential to exhibit increased oral bioavailability. This was demonstrated for both backbone modifications (methylphosphonates) and for sugar-modified (2′-*O*-methyl) oligonucleotides (141,203). We have found that 2′-*O*-methoxyethyl-modified oligonucleotides also exhibit increased oral absorption compared with phosphorothioate oligodeoxynucleotides (80,142).

The physicochemical properties of phosphorothioate oligodeoxynucleotides present a significant barrier to their GI absorption into the systemic circulation or the lymphatics. These factors include their large size and molecular weight (i.e., up to 6.5 kDa for 20-mers), hydrophilic nature (log $D_{o/w}$approximating -3.5) and multiple ionization pk_as (e.g., G. Hardee, 1999, unpublished titration data, using a Sirius GlpKa instrument on a 20-mer sequence, noted over 17 pk_as for phosphorothioate oligodeoxynucleotide and over 32 pk_as for the 2′-*O*-methoxyethyl hemi-mer form). The use of formulations can improve upon GI permeability. Oligonucleotide drug formulations designed to improve oral bioavailability need to consider the mechanism of oligonucleotide absorption—either paracellular via the epithelial tight junctions, or transcellular via direct passage through the lipid membrane bilayer. By using paracellular and transcellular models appropriate for water-soluble, hydrophilic macromolecules, it was determined that oligonucleotides predominantly traverse GI epithelium via the paracellular route. In this regard, formulation design considerations involve the selection of those penetration enhancers (PEs) that facilitate paracellular transport and meet other formulation criteria, including suitable biopharmaceutics, safety considerations, manufacturability, physical and chemical stability, and practicality of the product configuration (i.e., regarding production costs, dosing regimen, and patient compliance, etc.). Work is in progress, optimizing oligonucleotide chemistry with various permeation enhancers (142,204,205). Preliminary data are encouraging and support continued investment of resources on this endeavor.

E. Liposome Formulations

Liposome formulations of antisense oligonucleotides offer several potential advantages over saline phosphorothioate oligodeoxynucleotides, such as decreased toxicity, altered tissue and cellular distribution, and more convenient dose schedule for the patient. Interesting progress has been reported regarding the passive targeting of oligonucleotides to specific tissues using liposome-encapsulated therapeutics. Accumulation at sites of infection, inflammation, and tumor growth has been attributed to increased circulation times of these materials and the leaky vasculatures associated with these processes (206,207). One caution regarding these observations is worth noting. Because the mononuclear phagocyte system (MPS) is largely responsible for clearing these materials from circulation, misleading data regarding circulation time may be obtained in species with less-evolved systems (i.e., rodents).

Cationic liposomes bind to oligonucleotides due to the electrostatic interaction between positively charged head

groups on lipids and negatively charged phosphates on oligonucleotides. Using the technique of complexation, all the oligonucleotide can be entrapped and purification is not required. The utility of in vivo delivery of oligonucleotide using cationic lipid is limited due to sequestration of material in lung and the RES system (144,208). In addition, interaction of the complex with blood components leads to serum sensitivity and cytotoxicity (209,210).

There are few examples of oligonucleotide delivery by anionic or charge-neutral liposomes. Oligonucleotides encapsulated into cardiolipin-containing anionic liposomes were shown to be taken up 7 to 18-fold more in human T leukemia and ovarian carcinoma cells in vitro. The intracellular release of oligonucleotides was also facilitated and the majority of oligonucleotide was delivered into liposomes (211,212). Methylphosphonate analogs were incorporated into DPPC-containing liposomes and targeted against the Bcr-abl neogene found in chronic myelogenous leukemia (CML). The liposomal- encapsulated oligonucleotides inhibited the growth of CML cells (213). Cellular uptake of oligonucleotides against epidermal growth factor (EGF) encapsulated in DPPC:CHOL liposome containing folate was 9 times higher than nonfolate liposomes and 16 times higher than unencapsulated liposomes (214). There are 2 limitations to intracellular delivery of oligonucleotides by anionic or neutral liposomes: (1) not all cells take up particulate matter, and (2) these liposomes have low encapsulation efficiency.

There is only 1 report of using anionic liposomes in vivo to deliver oligonucleotides. Ponnappa et al. described liposomes consisting DPPC:CHOL:DMPG targeted toward Kupffer cells (215). In this study, greater than 65% of the liver-associated oligonucleotide was found in Kupffer cells.

Conjugation of antibodies to liposomes have been used for targeting of oligonucleotides to specific targets (216–220). Problems with the approach include the inhibition of cellular uptake by the high molecular weight antibody, cost, and poor encapsulation efficiency.

The primary mechanism for cell internalization of neutral liposomes is by endocytosis with the vesicles and their contents delivered to lysozomes (221). pH-sensitive liposomes have been designed to fuse with the endosomes at low endosomal pH and empty their content into cytosol. These pH-sensitive liposomes have been used to deliver antisense oligonucleotides. pH-sensitive liposomes composed of oleic acid: DOPE:Chol-encapsulating antisense oligonucleotide targeted against friend retrovirus inhibited the viral spreading, whereas free oligonucleotide and non pH-sensitive liposomes were ineffective (222,223). pH-sensitive liposomes encapsulating the anti-env oligonucleotide were found to inhibit viral spread at low concentration in infected Dunni cells (224). The major limitation of pH-sensitive liposomes in vivo is their instability in plasma (225,226). This problem was overcome by adding polyethylene glycol-phosphatidylethanolamine (PEG-PE) into the formulation (227). PEG-PE is believed to coat the surface of liposomes, thereby preventing the interaction of liposomes with blood components. This reduced interaction leads to increased stability and plasma half-life of liposomes. The pH-sensitive liposomes composed of CHEMS:DOPE: PEG-PE, when injected intravenously into rats, had similar pharmacokinetics parameters as non pH-sensitive sterically stabilized liposomes. The regular pH-sensitive liposomes without PEG-PE were cleared rapidly from the circulation.

Looking past the question of uptake, a novel approach to releasing endosomal contents into the cytoplasm after uptake has been recently reported (228–230). A 58-kDa protein isolated from Listeria monocytogenes was incorporated into pH-sensitive fluorescent dye. It could be determined that as soon as the endosome began to acidify, the liposome/endosome contents were released into the cytosol. As with the other delivery systems mentioned above, the eventual usefulness of a particular approach will be determined in the near future as we further define the mechanisms and governing restrictions for the inter- and intracellular trafficking of oligonucleotides.

VII. CLINICAL EXPERIENCE WITH ANTISENSE OLIGONUCLEOTIDES

More than 20 different antisense oligonucleotides are currently in clinical trials or approved for use in humans (Table 1). Similar to any other class of drugs, it can be expected that there will be failures in the clinic due to a variety of reasons, such as selection of the wrong molecular target resulting in lack of efficacy, incorrect dosing, marketing consideration, toxicity, etc. It is hoped that because of the generic pharmacokinetics and chemical class-specific toxicity that the failure rates for antisense oligonucleotides will be lower than other classes of agents. However, this remains to be seen.

A. Use of Antisense Oligonucleotides as Antiviral Therapy

The most advanced antisense product is Vitravene™ (fomivirsen, ISIS 2922), which is marketed in the United States for the treatment of patients with cytomegalovirus (CMV) retinitis. Fomivirsen was identified from a screen of a series of phosphorothioate oligodeoxynucleotides targeting human cytomegalovirus (HCMV) DNA polymerase gene, or to RNA transcripts of the major immediate-early regions 1 and 2 (IE1 and IE2) (231). Fomivirsen is a 21-mer phosphorothioate oligodeoxynucleotide targeting the coding region of the immediate early 2 gene. Fomivirsen inhibits viral protein expression, as measured by an enzyme-linked immunosorlent assay detecting an HCMV late protein product, in fibroblasts with an EC_{50} value of 0.1 μM. Noncomplementary phosphorothioate oligodeoxynucleotides exhibit an EC_{50} value of 2 μM, 20-fold higher than fomivirsen. In a plaque reduction assay, fomivirsen exhibited an IC_{70} value of 0.1 μM, whereas a control oligonucleotide exhibited an IC_{70} value of 2 μM. These data suggest that HCMV infection of human dermal fibroblast can be inhibited nonspecifically by higher concentrations of phosphorothioate oligodeoxynucleotides: however, fomivirsen is approximately 20-fold more effective than nonspecific oligonucleotides. Fomivirsen reduced IE1 and IE2 proteins in in-

fected cells, as did control oligonucleotides at 10-fold higher concentrations. As the IE1 and IE2 gene products arise from a common pre-mRNA, these results suggest that the oligonucleotide hybridizes to the pre-mRNA. Deletion of sequences from the 5′- and/or 3′-end of the oligonucleotides reduced antiviral activity, whereas introduction of mismatches in the interior of the oligonucleotide did not significantly reduce antiviral activity, although they did reduce hybridization to the target RNA. These data suggest that the antiviral activity of fomivirsen may not be due entirely to an antisense effect. To address this issue in more detail, U373 cells permanently transfected with the IE72 or IE55 polypeptides (derived from the IE1 and IE2 genes, respectively) were treated with fomivirsen (232). Fomivirsen reduced IE55 but not IE72 protein and RNA levels in a sequence-specific manner, suggesting that reduction of IE55 expression occurs by an RNase H-dependent mechanism. As the construct used to express IE72 protein does not contain the fomivirsen-binding site, these data would support that fomivirsen reduces IE55 expression by an antisense mechanism of action. The antiviral activity of fomivirsen was not due to immune stimulation by the CpG motifs in the oligonucleotide (167), as methylation of all the cytosines or only 2 cytosines in the CpG motifs did not reduce antiviral activity. These studies in aggregate suggest that fomivirsen is a potent inhibitor of CMV replication, which is capable of inhibiting viral gene expression by an antisense mechanism of action, but also may inhibit viral replication by a nonantisense mechanism of action at higher concentrations. Whether both mechanism of action are operational in the clinic remains to be elucidated.

Fomivirsen is approved for the local treatment of CMV retinitis in patients with acquired immunodeficiency syndrome, who are intolerant of or have a contraindication to other treatments of CMV retinitis (233,234). The recommended dose is 330 μg every other week for 2 doses and then a maintenance dose administered every 4 weeks given as an intravitreal injection. The most frequently observed adverse event reported for fomivirsen is ocular inflammation (uveitis), including iritis and vitritis (235). Ocular inflammation has been reported to occur in approximately 25% of the patients. Topical corticosteroids have been useful in treating the ocular inflammation. Open-label, controlled clinical studies have been performed, evaluating the safety and efficacy of fomivirsen in newly diagnosed CMV retinitis patients. Based on assessment of fundus photographs, the median time to progression was approximately 80 days for patients treated with fomivirsen, compared with 2 weeks for patients not receiving treatment (234). Although the market for CMV retinitis is relatively small, this drug represents an important validation for the technology.

Gem 132, a second-generation chimeric molecule targeting the HCMV UL36 gene product, is a 20-mer oligonucleotide containing two 2′-*O*-methyl nucleosides on the 5′-end of the molecule and four 2′-*O*-methyl nucleosides on the 3′-end, with the center 14 residues being oligodeoxynucleotides (236). The 2′-*O*-methyl residues also confer increased hybridization affinity and increased nuclease resistance, whereas the center oligodeoxynucleotide residues support RNase H activity. Gem 132 is being evaluated in CMV retinitis patients as both an intravenous infusion and as a direct intravitreal injection. In healthy volunteers, single 2-h infusions of GEM 132 were administered at doses ranging from 0.125 mg/kg to 0.5 mg/kg. Similar to phosphorothioate oligodeoxynucleotides, the plasma pharmacokinetics of GEM 132 were nonlinear with respect to dose. As a single dose up to 0.5 mg/kg, GEM 132 was well tolerated in normal volunteers, with headache being the most frequently reported side effect (237).

Gem 91, a 25-mer phosphorothioate oligodeoxynucleotide designed to hybridize to a conserved region of *gag* human immunodeficiency virus region of (HIV) RNA (238). GEM 91 inhibits viral replication in short-term viral assays in a concentration-dependent manner, whereas a 4- to 5-fold higher concentration of a random mixture of 25-mer phosphorothioate oligodeoxynucleotides (complexity = 4^{25} unique molecules) was required to inhibit viral replication to a similar extent (239). Other studies have demonstrated that acute HIV viral assays are particularly sensitive to the nonantisense effect of phosphorothioate oligodeoxynucleotides (240–243). In chronic HIV assays, GEM91 suppressed viral replication for greater than 30 days, whereas the random mixture of oligodeoxynucleotides only suppressed viral replication for 10 days. GEM 91 was found to be effective against several viral isolates in primary lymphocytes and macrophages, and exhibited selectivity in comparison to the random mixture. In that a random mixture of 4^{25} sequences was used as a control, it is difficult to conclude that GEM 91 inhibits viral replication in a sequence-specific manner. Based on these data, it is likely that at least part of the antiviral activity exhibited by GEM 91 is due to a nonantisense effect.

Phase I/II clinical studies were initiated for GEM 91 in the United States and France (236). The study performed in the United States was a randomized double-blind, placebo-controlled, dose-escalating study in which GEM 91 was administered as a continuous intravenous infusion for 2 weeks, whereas in the French study, GEM 91 was given as a 2-h intravenous infusion every other day for 28 days. Dose levels up to 4.4 mg/kg/day were achieved in the continuous infusion trials, whereas dose levels of 3.0 mg/kg/day were reported for the intermittent infusion trial. Plasma half-lives for GEM 91 were biphasic with mean half-lives of 0.18 h and 26.7 h (236,244). Hybridon recently announced the termination of clinical studies with GEM 91 based on lack of efficacy as measured by viral burden and the development of thrombocytopenia in some of the patients.

ISIS 14803 is a phosphorothioate oligodeoxynucleotide targeting the translation initiation codon of hepatitis C virus (245,246). ISIS 14803 differs from previous phosphorothioate oligodeoxynucleotides in that the cytosines were modified to 5 methyl cytosines, which further increases binding affinity for RNA and reduces the potential for immune stimulation (247). The oligonucleotide caused a reduction in target RNA, consistent with an RNase H mechanism of action and inhibited the production of hepatitis C viral proteins in hepatocyte cells transfected with a partial HCV genome containing the 5′-non-

coding region, core protein region, and the majority of the envelope region. HCV animal models were not readily available when this compound was being investigated preclinically. Therefore, a surrogate model was used to evaluate the potential in vivo efficacy of this oligonucleotide. A vaccinia virus model was used in which the HCV 5′-noncoding region, containing the IRES, and a portion of the core protein sequence was fused to firefly luciferase gene. Intraperitoneal injection of the recombinant vaccinia virus into mice, produced high levels of luciferase activity in livers. ISIS 14803 selectively inhibited luciferase expression in the livers isolated from infected mice (246). ISIS 14803 is currently in phase II trials, administered as an intravenous infusion, alone or in combination with interferon and ribavirin (248).

B. Use of Antisense Oligonucleotides for Cancer Therapy

An antisense oligonucleotide directed to p53 was one of the first antisense oligonucleotides to be administered systemically to patients. Preclinical studies with OL(1)p53, a 20-mer phosphorothioate oligodeoxynucleotide complementary to a portion in exon 10 of the p53 mRNA, inhibited proliferation of acute myelogenous leukemia cells in cell culture (136,249). Correspondingly, OL(1) p53 was found to reduce the level of p53 in leukemic cells, whereas a reverse sequence control failed to do so (136). A phase 1 study was conducted at the University of Nebraska Medical Center in which OL(1)p53 was infused at doses ranging from 0.05 mg/kg/h to 0.25 mg/kg/h for 10 days into patients with hematological malignancies. There were no apparent toxicities that could be directly attributed to the oligonucleotide. Two patients experienced a transient increase in hepatic transaminase concurrent with administration of the drug. In contrast to observations made with other phosphorothioate oligodeoxynucleotides, 17% to 59% of intact drug was detected in urine in this group of patients. There was an inverse correlation between plasma concentrations of oligonucleotide and cumulative leukemic growth of long-term marrow cultures. However, this correlation was not observed clinically as there were no morphological complete responses. These results provide evidence that OL(1)p53 was tolerated in leukemic patients; however, OL(1)p53 is no longer in active development.

Overexpression of *bcl*-2 is common in several cancers, in particular, non-Hodgkin lymphoma, and may contribute to decreased sensitivity to chemotherapeutic agents (250,251). An 18-mer phosphorothioate antisense oligodeoxynucleotide targeting the translation initiation codon of the *bcl*-2 gene was shown to inhibit the growth of lymphoma cells in severe combined immunodeficient (SCID) mice (252). Follow-up studies demonstrated that oblimersen inhibited growth of lymphoma cells in severely immunocompromised SCID and nonobese diabetic/SCID mice, suggesting that the activity of the oligonucleotide was not secondary to an immunostimulatory effect (253). The drug has also demonstrated antitumor activity in preclinical models of various other cancers such as melanoma, prostate cancer, and gastric cancer (252). Webb et al. conducted a phase 1 clinical trial of this oligonucleotide (Genta 3139, oblimersen) at the Royal Marsden Hospital in London. Genta 3139 was administered as a daily subcutaneous infusion for 14 days to patients with BCL-2 positive non-Hodgkin lymphoma. The dose of the drug given ranged from 4.6 mg/m^2 to 73.6 mg/m^2. Other than local inflammation at the site of infusion, no treatment-related side effects were noted. In 2 patients, tomography scans revealed reductions in tumor size with one complete response. In 2 additional patients, the number of circulating lymphoma cells decreased during treatment. Reduced levels of *bcl*-2 protein expression in circulating lymphoma cells were detected in 2 out of 5 patients. These findings again demonstrate that phosphorothioate oligodeoxynucleotides can be safely administered to patients and also provide preliminary efficacy data with a *bcl*-2 antisense oligonucleotide. Several other phase I/II studies on oblimersen have been performed, including studies in prostate cancer, breast cancer, colorectal cancer, AML, CML, multiple myeloma, and malignant melanoma (254,255). Side effects associated with the use of oblimersen included thrombocytopenia, hypotension, fever, and hypoglycemia (255). Promising clinical activity was seen in several of the studies, warranting continued investigation of the drug. The bcl-2 antisense oligonucleotide is currently in phase III trials for the treatment of melanoma, chronic lymphocytic leukemia, multiple myeloma, and non-small lung cancer, and in several additional phase II trials (255).

Protein kinase C (PKC) was originally identified as a serine/threonine kinase involved in mediating intracellular responses to a variety of growth factors, hormones, and neurotransmitters (256). Molecular cloning studies have revealed that PKC exists as a family of at least 11 closely related isozymes, which are subdivided on the basis of certain structural and biochemical similarities (257–260). Considerable experimental evidence exists for a role of PKC in some abnormal cellular process, such as inflammation, tumor promotion, and carcinogenesis (260–262). Antisense oligonucleotides have been identified that target individual members of the PKC family, both as research tools and as potential drugs (32,111,263,264). Protein kinase C-α has been implicated as a signaling molecule for a number of growth factors, and has been shown to regulate cell motility and apoptotic processes in human cells (265–271). To determine if inhibitors of protein kinase C-α could have potential value in the treatment of malignancies, we have identified an antisense oligonucleotide that specifically inhibit expression of PKC-α either in mouse or human cell lines (32,77,111). These antisense oligonucleotides have been used to identify cellular processes that are governed by this PKC isozyme (32,272–275).

The effects of the human-specific PKC-α phosphorothioate oligodeoxynucleotide, ISIS 3521/LY900003, has been examined on the growth of human tumor xenografts in nude mice. Analysis of PKC-α expression in the tumor tissue by immunohistochemistry revealed positive staining present in the cytoplasm and occasionally in the nuclei of tumor cells in animals treated with either saline or a scrambled control phosphorothioate oligodeoxynucleotide. In contrast, tumors treated with

ISIS 3521/LY900003 showed much reduced staining for PKC-α (276). In a second series of independent studies, ISIS 3521/LY900003 has been used to suppress the growth of U-87 glioblastoma tumor cells in nude mice (272). This cell line was chosen for study because it has previously been shown to be sensitive to growth inhibition by transfection with an antisense PKC-α cDNA. ISIS 3521/LY900003 reduced the growth of these tumor cells when implanted both subcutaneously and intracranially, whereas the scrambled control compound failed to inhibit tumor growth. This resulted in a doubling in median survival time of the animals with intracranially implanted tumors, with 40% long-term survivors of the treated animals. Levels of both ISIS 3521/LY90003 and the scrambled control oligodeoxynucleotide within tumor tissue were determined by capillary gel electrophoresis and found to both be about 2 μM after 21 daily intraperitoneal doses of 20-mg/kg oligodeoxynucleotide. ISIS 3521/LY900003 also reduced the expression of PKC-α in the tumor tissue, but not PKC-ε or PKC-ζ.

Based on the available biological evidence implicating PKC in the pathogenesis of certain tumor types and the broad spectrum of antitumor activity of ISIS 3521/LY90003 in the nude mouse xenograft implant model, clinical trials were initiated. A variety of tumors have been evaluated in phase I/II trials (277–280). In one trial, ISIS 3521/LY900003 was administered as a continuous 21-day infusion, then rested for 7 days. The cycle could be repeated if the treatments were tolerated and the tumor did not progress (277). The dose-limiting toxicities of ISIS 3521/LY90003 were thrombocytopenia and fatigue at a dose of 3.0 mg/kg/day. Pharmacokinetic measurements showed rapid plasma clearance and dose-dependent steady-state concentrations of ISIS 3521. Evidence of tumor response lasting up to 11 months was observed in 3 of 4 patients with ovarian cancer. There were no grade 3 or grade 4 toxicities reported. One patient displayed transient thrombocytopenia and 1 patient exhibited leukopenia. In a second phase I study, ISIS 3521/LY900003 was administered as a 2-h infusion 3 times per week for 3 consecutive weeks (278). A total of 36 patients received 99 cycles of the drug. Apparent drug-related toxicities included thrombocytopenia, nausea, vomiting, fever, chills, and fatigue. Dose escalation was stopped at a dose of 6 mg/kg because of concerns that peak plasma concentrations would approach those correlated with complement activation in monkeys (151). Most of the cancer patients had elevated baseline complement C3a. Following infusion of ISIS 3521/LY90003, several patients had a further increase in C3a; however, no clinical sequelae were attributed to the modest increases observed. Two non-Hodgkin lymphoma patients achieved complete responses, and 8 other patients showed stabilization of disease. Isis Pharmaceuticals, Inc., has completed several phase II trials including non-small cell lung carcinoma. Based on the phase II results, ISIS 3521/LY900003 is currently in 2 phase III trials for non-small cell lung carcinoma, one in combination with carboplatin and paclitaxel, and the second in combination with gemcitabine and taxol. ISIS 3521/LY9200003 is also being evaluated in additional phase II trials.

The discovery of viral oncogenes in the mid-1960s was a major breakthrough in understanding the molecular origins of cancer and directly led to the identification of the first human oncogene in 1982, ras (281). An antisense oligonucleotide targeting ha-ras gene product has initiated clinical trials. ISIS 2503 targets the AUG translation initiation codon for ha-ras gene product (282). Although the frequency of mutations in human cancers is significantly higher for the ki-ras gene product, we have found that antisense oligonucleotides targeting ha-ras gene exhibit broader antitumor effects when evaluated in human tumor xenograft models. In fact, the ha-ras antisense oligonucleotide was effective against human tumor xenografts known to contain a mutation in the ki-ras gene. A multicenter phase I trial against a broad spectrum of cancers has been completed (283). Patients received ISIS 2503 as a continuos intravenous infusion for 2 weeks, followed by a 1-week drug-free period. Patients will repeat the cycle as long as they tolerate the drug or when tumors fail to respond to therapies. In a second study, the drug was administered in a more convenient schedule (i.e., a weekly 24-hour infusion of ISIS 2503). Similar to the PKC-α and C-raf kinase antisense oligonucleotides, the drug was tolerated and exhibited enough, encouraging activity to warrant continuing phase II trials. Thus, a first-generation phosphorothioate oligodeoxynucleotide targeted to normal ha-*ras* is the first selective inhibitor of ras function to enter clinical trials.

Alterations in cellular cAMP concentrations have been associated with changes in cellular proliferation states. There are two isoforms of the major cAMP receptors, cAMP-dependent protein kinases I and II that are distinguished by different regulatory subunits (RI and RII). Increased expression of the RI subunit of PKA I correlates with cellular proliferation and cellular transformation, whereas a decrease in the RI subunit and an increase in the RII subunit correlates with growth inhibition and cellular differentiation (284). To directly address the role of the RI subunit in cell growth and differentiation, an antisense oligonucleotide targeting the RI subunit was designed. This oligonucleotide at concentration of 15 to 30 μM inhibited growth of several human cell lines without signs of cytotoxicity (285–287). As expected, the phosphorothioate oligodeoxynucleotide was more effective than the phosphodiester version. A single injection of the RI subunit phosphorothioate oligodeoxynucleotide suppressed growth of a human colon carcinoma xenograft for a week (286). Tumors exhibited normal growth rates when treated with a control oligonucleotide. Examining levels of PKA-I activity in the tumor xenografts provided further support of an antisense mechanism. The antisense oligonucleotide-treated tumors exhibited loss of enzyme activity 24 h after treatment. More recently, a second-generation, 2-*O*-methyl chimeric oligonucleotide (GEM231) targeting human PKA RI subunit has been described (288–290). This oligonucleotide was more effective than the first-generation oligonucleotide in suppressing growth of human tumor xenografts and has shown enhanced activity when combined with various chemotherapeutic agents. Clinical trials have been initiated with a 2′-*O*-methyl

chimeric PKA RI subunit antisense oligonucleotide (GEM 231) in the treatment of solid tumors.

C. Use of Antisense Oligonucleotides for Treatment of Inflammatory Diseases

In addition to targeting gene products implicated in viral replication or cancer, antisense oligonucleotides have been used to inhibit the expression of gene products, which may have utility for the treatment of inflammatory diseases. Intercellular adhesion molecule 1 (ICAM-1) is a member of the immunoglobulin gene family expressed at low levels on resting endothelial cells and can be markedly up-regulated in response to inflammatory mediators, such as TNF-α, interleukin 1, and interferon-γ on a variety of cell types. ICAM-1 plays a role in the extravasation of leukocytes from the vasculature to inflamed tissue and activation of leukocytes in the inflamed tissue (291–294). Alicaforsen (ISIS 2302) was identified out of a screen of multiple first-generation phosphorothioate oligodeoxynucleotides targeting various regions of the human ICAM-1 (2,295). Alicaforsen inhibits ICAM-1 expression by an RNase H-dependent mechanism of action (295). Alicaforsen will selectively inhibit ICAM-1 expression in a variety of cell types (295–297). Both sense and a variety of scrambled control oligonucleotides fail to inhibit ICAM-1 expression, including a 2-base mismatch control. Treatment of endothelial cells with alicaforsen blocked adhesion of leukocytes, demonstrating that blocking expression of ICAM-1 will attenuate adhesion of leukocytes to activated endothelial cells (295). ISIS 2302 also blocked a 1-way mixed lymphocyte reaction when the antigen-presenting cell was pretreated with ISIS 2302 to down-regulate ICAM-1 expression prior to exposure to the lymphocyte (Vickers et al., 1996 unpublished data). Thus, alicaforsen is capable of blocking both leukocyte adhesion to activated endothelial cells and costimulatory signals to T lymphocytes, both activities were predicted based on previous studies with monoclonal antibodies to ICAM-1.

To test the pharmacology of the human-specific antisense oligonucleotide, we have used experimental models in which immunocompromised mice contain human tissue xenografts. In one model, we were able to demonstrate a role for ICAM-1 in metastasis of human melanoma cells to the lung of mice (296). A second study addressed the role of ICAM-1 in production of cytotoxic dermatitis (lichen planus) in SCID mice containing human skin xenografts (298). Upon engraftment of the human tissue, heterologous lymphocytes injected into the graft migrate into the epidermis (epidermaltropism), and produced a cytotoxic interaction between effector lymphocytes and epidermal cells. Systemic administration of alicaforsen inhibited ICAM-1 expression in the human graft, decreased the migration of lymphocytes into the epidermis, and prevented subsequent lesion formation. A sense control oligodeoxynucleotide failed to attenuate the responses. These data demonstrate that an ICAM-1 antisense oligonucleotide administered systemically can attenuate an inflammatory response in the skin.

ISIS 3082 and ISIS 9125 are 20-base phosphorothioate oligodeoxynucleotides that hybridize to an analogous region in the 3′-untranslated region of murine and rat ICAM-1 mRNA, respectively (299,300). Similar to alicaforsen, ISIS 3082 and ISIS 9125 selectively inhibit ICAM-1 expression in mouse or rat cells by an RNase H-dependent mechanism. Rodent ICAM-1 antisense oligonucleotides have demonstrated activity in a mouse heterotopic heart transplant model (299), mouse pancreatic islet transplant model (301), and rat heart and kidney transplants (300). The murine ICAM-1 antisense oligonucleotide has also shown activity in mouse models of pneumonia, colitis, and arthritis (165,302). Haller et al. independently used an ICAM-1 antisense oligonucleotide to decrease acute renal injury following ischemia in rats (303).

Alicaforsen, which targets human ICAM-1, is currently being developed by Isis Pharmaceuticals, Inc., for the treatment of Crohn's disease and ulcerative colitis. Safety and pharmacokinetics of alicaforsen was established in a phase I study performed at Guy's hospital in normal volunteers (130). Volunteers were either infused over a 2-h period with escalating single doses or multiple doses given of alicaforsen or saline in a double-blinded trial. Brief dose-dependent increases in aPTT were seen at the time of peak plasma concentration and clinically insignificant increases in C3a were seen after repeated 2.0 mg/kg doses. C5a, blood pressure, and pulse were unaffected by administration of alicaforsen. No other adverse events or laboratory abnormalities related to the administration of the drug were noted. The Cmax was linearly related to dose and occurred at the end of infusion. Plasma half-life was approximately 53 min. Nonlinear changes in AUC and volume of distribution were noted with increasing dose, suggesting that oligonucleotide disposition might have a saturable component. These data suggest that ISIS 2302 was well tolerated in normal volunteers and that the pharmacokinetics in man was similar to that observed in nonhuman primates and rodents.

Alicaforsen was evaluated in a series of small phase IIa studies (20–40 patients in each trial) in rheumatoid arthritis, psoriasis, Crohn's disease, ulcerative colitis, and renal transplant. With the exception of the psoriasis study, the trials were placebo-controlled, double-blinded trials in which the drug is administered as a 2-h intravenous infusion. In all trials, the drug was well tolerated. In the rheumatoid arthritis trial, alicaforsen failed to produce significant efficacy but showed positive trends (304). The small sample size of the trial, 43 patients, did not allow definitive conclusions to be drawn.

In the phase IIa Crohn's disease study, conducted by Dr. Bruce Yacyshyn at the University of Edmonton, patients were administered 0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg ISIS 2302 every other day for a total of 26 days (305). The response of the patients was not dose dependent, probably due to the narrow dose range investigated and the small number of patients in the lower dose groups (3 each). Therefore, all ISIS 2302-treated patients were analyzed as 1 group. Complete response, defined as Crohn's disease activity index (CDAI) score less than 150, was observed in 7 of 15 patients treated with ISIS 2302 and 0 of 4 of the placebo patients (305). At the end of

the study (6 months), 5 of the 7 patients were still in remission and 1 patient had a CDAI score of 156. During the treatment phase of the study, steroid doses were fixed, afterward the physician was allowed to adjust steroid dose based on symptoms. There was a statistically significant decrease in steroid use in patients treated with ISIS 2302 compared with placebo-treated patients at the end of the study. Other than expected increase in aPTT and mild facial flushing at the end of infusion in 1 patient, the drug was well tolerated. Based on these promising data, a large multicenter phase IIb trial of ISIS 2302 in Crohn's disease has been initiated. Thus, ICAM-1 antisense oligonucleotides may have therapeutic utility for the treatment of Crohn's disease.

The pilot trial in Crohn's disease was followed with a larger 299-patient, placebo-controlled, multicenter trial (306). Alicaforsen was administered intravenously 3 times per week at a dose of 2 mg/kg for either 2 weeks or 4 weeks. Patients were treated in months 1 and 3, with steroid withdrawal attempted by week 10. The primary endpoint of the trial was a CDAI less than 150 and off steroids at the end of week 14. Of the patients completing week 14, 64% of placebo patients had discontinued steroids and 78% of the alicaforsen-treated patients had successfully stopped steroid use ($p = 0.032$) (306). The number of patients achieving steroid-free remissions was similar in all 3 treatment arms. Pharmacodynamic analysis revealed that there was a strong correlation between drug exposure as measured by plasma AUC levels and response to alicaforsen treatment. Remissions increased from 13.0% (7/54) for the lowest AUC group to 55.6% (5/9) for the highest AUC group. One patient in the trial developed an IgM antibody to alicaforsen, without clinical sequelae. Adverse events reported in the trial were minimal with 2% of the patients exhibiting hypersensitive reactions. Anticipated increase in aPTT without bleeding episodes were noted and transient facial flushing during infusion were also noted. Although the trial was not positive, the correlation between exposure and clinical response warranted further investigation. As such, a 22-patient safety and pharmacokinetic trial was recently completed examining 250, 300, and 350 mg doses of alicaforsen. Doses were based on body weight. Infusion-related reactions of fever, chills, headache, nausea, emesis, and arthralgias were reported in 41% of the patients. Infusion-related reactions appear to be less frequent in patients receiving background steroids. The pharmacokinetic data suggest that patients receiving 300 to 350 mg of alicaforsen achieved adequate drug exposure, and this dose is currently under evaluation in a phase III trial.

Inhibitors of TNF-α have proven clinically useful for the treatment of rheumatoid arthritis, psoriasis, and Crohn's disease (307–311). Antisense oligonucleotides to TNF-α have demonstrated positive effects in mouse colitis models and a mouse model of stroke (312,313). ISIS 104803 is a second-generation chimeric, 2′-*O*-methoxyethyl/oligodeoxynucleotide targeting human TNF-α (116). A phase I study of ISIS 104803 has been completed in healthy males (116). The drug was dosed from 0.1 to 6.0 mg/kg given either intravenously of subcutaneously, with up to 4 doses given. Transient prolongation in aPTT was observed, similar to first-generation phosphorothioate oligodeoxynucleotides. Two patients experienced a rash, 1 a reversible platelet decrease, and tenderness was noted at the site of a subcutaneous injection. A decrease in TNF-α production was noted in peripheral blood leukocytes activated ex vivo with endotoxin in subjects treated with ISIS 104803. ISIS 104803 is currently under investigation in phase II trials for rheumatoid arthritis and psoriasis.

VIII. CONCLUSION

As is to be expected with first-generation technology, undesirable properties have been identified for phosphorothioate oligodeoxynucleotides (149,150,314,315). Despite these limitations, it is possible to use phosphorothioate oligodeoxynucleotides to selectively inhibit the expression of a targeted RNA in cell culture and in vivo. The pharmacokinetics of phosphorothioate oligodeoxynucleotides are similar across species and do not appear to exhibit major sequence-specific differences. When dosed at high levels, it is possible to identify toxicities in rodents and primates. However, at doses currently under evaluation in the clinic, phosphorothioate oligodeoxynucleotides have been well tolerated. In addition, there is evidence that phosphorothioate oligodeoxynucleotides provide clinical benefits to patients with viral infections, cancer, and inflammatory diseases. There are several phosphorothioate oligodeoxynucleotides in late-stage clinical trials, which will hopefully deliver more effective therapies for patients suffering from life-threatening or very debilitating diseases.

Extensive medicinal chemistry efforts have been successfully focused on identifying improved antisense oligonucleotides, which address some of these issues. There are at least 4 areas in which chemistry can add value to first-generation drugs: increase potency, decrease toxicity, alter pharmacokinetics, and lower costs. As an example, numerous modified oligonucleotides have been identified that have a higher affinity for target RNA than phosphorothioate oligodeoxynucleotides (84,87,91–93,316). Oligonucleotide modifications have been identified that exhibit increased resistance to serum and cellular nucleases, enabling use of oligonucleotides that do not have phosphorothioate linkages. The tissue distribution of oligonucleotides may be altered with either chemical modifications or formulations (79,134,140,141,143,144,181,200,203). Preliminary data also suggest that oral delivery of antisense oligonucleotides may be feasible (141). Finally, a number of modified oligonucleotides have been described that potentially exhibited less toxicities than first-generation phosphorothioate oligodeoxynucleotides (78,149,178)]. However, as experience with these modified oligonucleotides is rather limited, it remains to be seen whether they will have a distinct toxicity profile.

In conclusion, first-generation phosphorothioate oligodeoxynucleotides have proven to be valuable pharmacological tools for the researcher and have produced new therapies for the patient. Identification of improved second-and third-generation oligonucleotides with novel formulation should better

therapies for patients. Although tremendous progress has been made for antisense technology during the past 14 years, there are many more questions that remain for the technology.

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20

Suicide Gene Therapy

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I. INTRODUCTION

Despite decades of scientific advancements and new developments in surgery, radiation therapy, and chemotherapy, human cancer continues to be a major and growing cause of death in the world today. For most solid tumors, surgical excision is the primary mode of treatment and is frequently combined with radiation or chemotherapy for maximal efficacy. Radiation is capable of destroying both normal and abnormal tissues, but has a preferential effect on dividing malignant cells. Chemotherapy involves the use of cytotoxic drugs, which also have a preferential effect on rapidly dividing cells. Chemotherapeutic drugs are often used in combination because tumors have shown greater susceptibility to multiple agents that attack tumor cells by different mechanisms. Progress with these standard modalities for treating cancer continues while a new field of cancer research and treatment is evolving at a rapid pace. This new field is ''gene therapy'' and is founded on the premise that specific genes can be introduced into tumor cells to cause either a direct or indirect antitumor effect.

At first consideration, cancer does not appear to be a suitable target for ''classic gene therapy.'' That is, the original concept of gene therapy stemmed from the observation that certain diseases are caused by the inheritance of a single defective gene. Such diseases with monogenic defects such as adenosine deaminase deficiency and Gaucher's disease could theoretically be treated by effective insertion and expression of a normal gene into the defective host cells or even the bone marrow stem cell population (1,2). However, our recent understanding of cancer is that it develops via a multistep process whereby a series of genetic alterations or mutations occur within 1 cell (3). This 1 cell then looses regulatory control and proliferates abnormally to produce a population of malignant cells that may also continue to mutate and lead to cancer spread or metastases. Given the complexity of this genetic progression of cancer and the still many unknown abnormalities within any given tumor type, the idea of replacing all the abnormal genes with normal copies using gene therapy would be prohibitive. Also, with the limitations of delivery vehicles or ''vectors'' currently available for gene therapy, it would be equally prohibitive to effectively deliver the normal genes to every defective cancer cell.

Cancer gene therapy, however, is moving in directions that attempt to circumvent these apparent limitations. On such direction is the development of prodrug or ''suicide gene therapy'' strategies that involve specific gene transfer into tumor cells that enables intracellular conversion of a ''nontoxic'' prodrug into an active cytotoxic drug. Following effective suicide gene transfer and systemic or oral administration of the prodrug, an enzyme encoded by the therapeutic gene converts the prodrug into toxic anabolites or metabolites that inhibit or disrupt DNA synthesis. The tumor cells subsequently die via necrosis or apoptotic pathways. The most common suicide gene used in preclinical and human clinical investigation is the gene encoding herpes simplex virus thymidine kinase (HSV-tk). A major advantage of many suicide gene therapy strategies is that not only are the tumor cells that have incorporated the gene destroyed, but also surrounding ''nontransduced'' cells are killed (4). The mechanism by which nontransduced neighboring tumor cells are killed is known as the ''bystander effect,'' which is discussed in more detail later in the chapter. The many facets of the bystander effect significantly strengthen suicide gene therapy as a whole and add to its versatility.

Although the identification and selection of specific suicide genes that may induce both direct and/or indirect antitumor effects is a very important area in gene therapy research, equally important to the success of such strategies is the grow-

ing arena of vector or delivery vehicle development. The choice of delivery vehicle greatly influences the overall outcome and efficiency of the therapeutic strategy. Even with the beneficial bystander effect, the effects of suicide gene therapy are linearly related to the efficiency of gene transfer. New investigations and advances in both viral and nonviral gene therapy technologies should also prove valuable in overcoming many of the obstacles in the application of gene therapy to cancer. One such recent advance is the application of replication-selective oncolytic viruses. These viruses have been engineered to selectively infect and replicate in targeted tumor cells that have inherent genetic defects, such as loss of p53 gene expression. Tumor cells that lack p53 expression allow select viral replication, which kills the host tumor cell, and then subsequent spread and infection of surrounding tumor cells results in further tumor kill. This modification of classic suicide gene therapy into suicide viral therapy may prove a key advance in overcoming limitations of gene transfer efficiency in the presently available replication-incompetent adenoviral vectors.

This chapter describes the various suicide gene therapy strategies, including a brief overview of replication-selective oncolytic virotherapy for treating solid tumors, and provides an overview of advantages and disadvantages of viral vs. nonviral methods of delivering these genes. The 2 basic approaches for suicide gene delivery ''in vivo and ex vivo'' are also reviewed, and an update on the status of human clinical trials is provided.

II. GENERAL PRINCIPLES AND SAFETY ISSUES

The principle of suicide gene therapy stems from the long history of drug discovery and development focusing on the treatment of microbial infections. The enyzmes encoded by suicide genes are important components in metabolic conversion pathways within bacteria, viruses, or fungi (5). The effect of a prodrug on human cells that have been transduced with a suicide gene therefore parallels the effect of an antibiotic on a human bacterial infection. The microbial origin of these enzymes provide 2 important safety and specificity features of suicide gene–prodrug therapy that are similar or identical to the features of antibiotic or antiviral therapy. First, the enyzmes and the associated metabolic pathway may be completely specific to microbial cells and not found in mammalian cells. Because mammalian cells lack the enzyme encoded by the suicide gene, systemic administration of the prodrug has no toxic effect on any human tissue or cell that has not been engineered to express the selected suicide gene (6). Second, certain mammalian cells may actually express the enyzme, but the specific prodrug chose may be a poor substrate for the mammalian form of the enzyme and an excellent substrate for the bacterial or viral form. Again in this scenario, administration of the prodrug would be harmless to the mammalian cell that has not been engineered to overexpress the nonmammalian enzyme. The prodrug is therefore similar to or may actually be a form of antibiotic or antiviral medicine. Systemic administrations of the prodrug will produce effects limited to cells expressing the suicide gene and the microenvironment surrounding these cells and would not cause significant systemic toxicity.

Another general safety feature for suicide gene therapy lies within the actual mechanism of cytotoxicity that results from the conversion of the prodrug to a toxic metabolite. Because the majority of these metabolites inhibit cellular nucleic acid synthesis pathways, the delivery of the suicide gene and administration of the prodrug would have a preferential cytotoxic affect on actively dividing cells. With respect to tumor cell populations vs. normal mammalian cells, the tumor would be more susceptible to the effects of suicide gene therapy. Some suicide gene therapy systems do not show preference for dividing cells, which raises a safety issue. Surrounding normal tissue may be inadvertently injured after exposure and subsequent transduction by the suicide gene. Selection and application of gene delivery techniques are therefore important in targeting the tumor while minimizing exposure to normal or systemic tissues to the suicide gene.

III. SUICIDE GENE THERAPY STRATEGIES

A key issue in the success of a suicide gene therapy strategy is the interaction between the enzyme produced and the prodrug administered. The selection of the enzyme and prodrug combination is influenced by certain variables critical to enyzme-substrate kinetics. Two important variables exist for the enzyme. The first is the speed of activation of the prodrug. The most effective suicide genes will express enzymes that rapidly activate the prodrug. Enzymes that are slower in their activation will be dependent on either higher concentrations of the prodrug or prolonged administration. Issues such as half-life and intracellular degradation and clearance mechanisms will also limit the presence of the prodrug. The second variable is the efficiency of prodrug activation. Enzymes that are highly efficient in converting the prodrug substrate into its toxic metabolite should prove advantageous because of known variances in the levels of effective gene transfer and gene expression (enzyme production) inherent to in vivo suicide gene transfer. Regarding the prodrug, it should be at least 100 times more cytotoxic than the preactivated form upon enzyme activation (7). Because many different types of prodrugs can be designed to achieve maximal activation, the most important and probably most limiting factor is the enzyme and therefore choice of suicide gene. Table 1 depicts the more commonly used suicide genes and corresponding prodrug combinations under investigation for gene therapy application. Table 2 shows the mechanisms of toxicity for selected genes that are presently being investigated in both preclinical and clinical studies.

Despite choosing a generally efficient enzyme–prodrug combination that proves successful with in vitro experimentation, there may not be a paralleled success after the transition to in vivo application against established tumors. In vivo effi-

Table 1 Selected Suicide Gene Therapy Strategies

Gene/enzyme	Prodrug	Initial toxic metabolite
Herpes simples virus thymidine kinase (HSV-tk)	Ganciclovir (GCV)	Ganciclovir monophosphate
Cytosine deaminase (CD)	5-Fluorocytosine (5-FC)	5-Fluorouracil (5-FU)
Varicella zoster virus thymldine kinase (VZV-tk)	6-Methoxypurine arabinonucleoside (araM)	Adenine arabinonucleoside monophosphate
Escherichia coli nitroreductase (NTR)	5-(Aziridin-1-yl)-2, 4-dinitrobenzamide (CB 1954)	5-(Aziridin-1-yl)-4-hydroxyamino-2-nitrobenzamide
Cytochrome P450 B1 (CYP2B1)	Cyclophosphamide (CPA)	4-Hydroxy-cyclophosphamide (4-HCPA)
Carboxypeptidase G2 (CPG2)	Benzoic acid mustard gluonuridebenzoic acid mustard (CMDA)	
E. coli gtp (XGPRT)	6-Thioxanthine (6-TX)	6-Thioxanthine monophosphate (6-XMP)
E. coli Deo (PNP)	6-Methyl purine deopxyriobside (MePdR)	6-Methylpurine (MeP)

cacy may also vary among different types or classes of tumors for any one given enzyme–prodrug combination. An example of this is found within the HSV-tk gene therapy strategy, the most widely used suicide gene in both preclinical investigation and human clinical trials. Although HSV-tk has proven effective in many different solid tumors, it is generally less effective against hematopoietic malignancies (8). It has been hypothesized that HSV-tk is down-regulated or lost more quickly in hematopoietic tumors such as leukemia, which results in ineffective or insufficient conversion of the prodrug to achieve antitumor effects (9).

A. HSV-tk

HSV-tk has shown the greatest potential to date for human application across a broad range of malignancies, and for this reason it is the most popular and widely studied suicide gene therapy strategy. The importance of HSV-tk gene transfer centers on its ability to render cells sensitive to the acyclic guanosine analog ganciclovir (GCV) (10,11). HSV-tk is a prototype ''suicide gene'' because it encodes a viral enzyme that is foreign to mammalian cells and will convert an inactive and relatively nontoxic prodrug to a toxic product. Upon effective HSV-tk gene transfer and expression, the prodrug GCV is monophosphorylated by the enzyme. Intracellular host kinases then metabolize this monophosphorylated nucleoside analog into di- and triphosphates (12). The triphosphate form of GCV is then incorporated into the replicating DNA chain in dividing cells and inhibits DNA polymerase. Inhibition of DNA polymerase results in chain termination, disruption of DNA synthesis, and cell death. The phosporylation of GCV impairs its ability to cross the cell membrane and, as a result, the half-life increases by 6-fold to 18 to 24 h (13,14). The extended half-life of the phosphorylated GCV strengthens the overall anticancer effect of this HSV-tk strategy. With respect to sensitivity, viral thymidine kinase is approximately 1000 times more efficient in phosphorylating GCV than its mammalian counterpart (13). Because GCV is an excellent substrate for

Table 2 Mechanisms of Cytotoxicity of Select Suicide Gene Therapy Strategies

Gene	Final toxic metabolite(s)	Direct cytotoxic effect	Bystander effect
HSV-tk	GCV-triphosphate	Disrupts DNA synthesis	Present
CD	5-FU-mono/tripohosphate	Disrupts DNA and RNA synthesis	Present
VZV-tk	Ara-ATP	Disrupts DNA synthesis	Unknown
NTR	4-Hydroxylamine metabolite of CB 1954	Cross-links DNA (direct DNA breaks; disrupts synthesis)	Unknown
CYP2B1	Phosphoramide mustard DNA acrolein	Alkylation (cross-links DNA) covatently links cellular proteins	Present
CPG2	Benzoic acid mustard	DNA alkylation (cross-links DNA)	Present
XGPRT	6-Thioguanine monophosphate (6-GMP)	Disrupts DNA synthesis	Presumed
PNP	6-Methylpurine (MeP)	Direct toxin/cellular necrosis	Present

viral thymidine kinases and a poor substrate for mammalian thymidine kinases, concentrations can be achieved that are lethal to cells expressing HSV-tk but are nontoxic to normal mammalian cells (10,11,15).

Culver et al. demonstrated the first in vivo application of suicide gene therapy for cancer using retroviral-mediated HSV-tk gene transfer via fibroblast packaging cells injected into brain tumors in mice (16,17). Since this initial study, HSV-tk suicide gene therapy has been applied and investigated in multiple tumor types, including thoracic, head and neck, and ovarian cancer (18–20). Critical to the tumor response described in these early studies was the observation that not all the tumor cells must express the HSV-tk gene for a complete or extensive tumoricidal effect. The term bystander effect has been attributed to the regression of noninfected surrounding tumor cells after HSV-tk delivery and GCV administration. There are both direct and indirect mechanisms driving the bystander effect that are discussed in more detail later in the chapter. Briefly, the direct effect is an exchange of toxic metabolites between HSV-tk infected tumor cells and neighboring noninfected cells (21,22). The indirect effect stems from antitumor immune responses add to the versatility of this suicide gene therapy strategy (23,24).

A potential disadvantage of the HSV-tk strategy is that it requires S-phase cell cycle activity and thus targets only dividing cells. At any given time, not all malignant cells within a tumor are cycling and different tumor types display different rates of cell doubling both in vitro and in vivo. Conditions must therefore be worked out that provide adequate HSV-tk gene expression and administration of GCV over long enough time to account for variability in cell cycling within a designated tumor. Although the bystander effect helps augment the antitumor effects, there is likely a threshold of HSV-tk transfer and expression and persistence of the enzyme that must be achieved to generate or sustain a significant therapeutic effect.

Although a potential disadvantage, the requirement for cell division may also be an advantage for both safety and tumor targeting for the HSV-tk strategy. Normal mammalian cells in general divide at much slower rates than tumor cells within an established malignancy. This differential in S-phase activity allows for design of HSV-tk delivery and expression that would preferentially kill tumor cells while minimizing or eliminating direct toxicity to surrounding or systemic normal tissues that may incorporate the gene and express the enzyme.

B. Cytosine Deaminase

The enzyme cytosine deaminase (CD) is found in a variety of fungi and bacteria, but is not found in mammalian cells. In these microbes, CD is activated during nutritional stress and normally catalyzes the deamination of cytosine to produce uracil. CD is also capable of converting the nontoxic prodrug 5-fluorocytosine (5-FC) into the metabolite 5-fluorouracil (5-FU) (24–26). In a second metabolic conversion process, intracellular enyzmes present in both microbial and mammalian cells then act on 5-FU to produce 5-fluorouridine 5′-triphosphate and 5-fluoro-2′-deoxyuridine 5′-monophosphate. These toxic phosporylated metabolites disrupt both RNA and DNA synthesis resulting in direct cellular cytotoxicity. Because of the natural specificity of CD to fungi and relative lack of toxicity of 5-FC in human tissues at routine dosing, 5-FC has been developed as an antifungal agent. However, because 5-FU is also converted to toxic metabolites in mammalian cells, this compound has been developed and widely applied as a chemotherapeutic agent for cancer.

The original application of CD for cancer therapy involved implanting a capsule containing the enzyme into an established rat tumor whereupon the enzyme diffused into the tumor cells. The animals were subsequently treated with 5-FC, which resulted in an antitumor response (27). Upon the cloning of the gene and building on these early animal investigations, CD suicide gene therapy became a feasible strategy (24). Aside from the direct cytotoxicity with this system, a significant bystander effect has also been identified with this system. The CD system is different from the HSV-tk system and may have a bystander advantage because of the initial production of 5-FU. Because the initial conversion to a ''toxic metabolite'' (5-FC to 5-FU) does not involve phosporylation, the 5-FU produced within the cytoplasm may readily cross the cell membrane and enter surrounding tumor cells. Also, the phosphorylated toxic metabolites of 5-FU that disrupt DNA and RNA synthesis may also be transferred to surrounding cells via intercellular communication or similar mechanisms as reported for the HSV-tk system. In effect, the multiple conversion steps provide 2 possible means of bystander activity that theoretically provide an enhanced overall antitumor and bystander effect as compared with the HSV-tk system. The full efficiency of the CD strategy and its bystander effect, however, has not been elucidated and further preclinical investigations are needed to discern any significant bystander advantages.

The lack of natural CD within mammalian cells and safety of 5-FC dosing makes this enzyme–prodrug combination a natural choice for human cancer investigation. CD is presently the second most common suicide gene under preclinical and clinical investigation; however, it has certain limitations that may affect successful application of this strategy. The toxicity of 5-FU is not S-phase cell-cycle specific, but it does depend on cell proliferation for its effect. As with the HSV-tk system, the CD strategy preferentially affects tumor cells that have a higher rate of proliferation. Although this provides some inherent safety and tumor targeting specificity, heterogenous tumor cell populations with variable levels of cellular proliferation may reduce the overall therapeutic effect. To achieve substantial tumor cell toxicity, high doses of 5-FU at the cellular level are generally required. The need for high cellular levels of 5-FU requires both efficient CD gene transfer and expression followed by adequate systemic dosing of 5-FC. The need for higher doses of 5-FC prodrug introduces the potential for systemic intestinal toxicity. The reason for this potential intestinal side effect is that residing enterobacteria present in normal human gastrointestinal tissues produce CD. These normal intestinal bacteria thus are able to convert 5-FC to 5-FU and produce local cellular injury. Another major

limiting factor is that 5-FU is only active for a maximum of 10 min. Thus, prolonged systemic treatment with 5-FC after CD gene transfer is required to maintain the production of intracellular 5-FU. Possibly the most limiting factor is the complexity of the metabolic conversion pathway itself. A multistep complex pathway provides many opportunities for tumor cells to acquire resistance to the actual therapy (28).

C. Varicella Zoster Virus Thymidine Kinase

The varicella zoster virus is also capable of expressing a unique thymidine kinase (VZV-tk) whose substrate specificity is distinct from both mammalian cellular kinases and the HSV-tk enyzme. Upon gene transfer of VSV-tk into recipient cells, cytotoxicity is induced by administration of the prodrug 9-(b-D-arabinofuranosyl)-6-methoxy-9H-purine, also known as araM (29). VZV-tk enyzme initially phosphorylates araM that is further metabolized by natural cellular enzymes (AMP deaminase, AMP kinase, nucleoside diphosphate kinase, and adenylosucinate synthestase lysase) into adenine arabinonucleoside triphosphate (araATP). ara-ATP is highly toxic and therefore only small quantities of ara-M in the range of 1 to 100 μm are required to directly kill cells that contain the VZV-tk enzyme (29). As with GCV in the HSV-tk system, araM is an excellent substrate for VZV-tk but not mammalian nucleoside kinases. Normal mammalian cells are able to withstand over 1500 μm of ara-M exposure. The overall sensitivity of a transduced cell to the ara-M prodrug is directly proportional to level of the VZ-tk activity.

This system is still relatively new for preclinical investigation, and the presence of a bystander effect remains to be proven. The disadvantages of VZV-tk suicide gene therapy are similar to those of HSV-tk, and further experimentation is required to define the efficacy and safety rationale for selecting between these two strategies.

D. *Escherichia coli* Nitroreductase

The NTR enzyme activates the relatively nontoxic prodrug dintrophenylaziridine CB1954 through a reduction process that generates a 4-hydroxylamine metabolite. This intermediate molecule further reacts with intracellular thioesters such as acetyl-CoA to produce a highly cytotoxic alkylating agent that is 10,000 times more toxic than the original prodrug (30).

There are a few proposed mechanisms by which CB1954 mediates its cellular toxicity after reduction by nitroreductase (NTR). The most commonly reported mechanism is through cross-linking DNA strands causing disruption of synthesis and DNA breaks that are directly cytotoxic to both dividing and nondividing cells (31). Some investigators have reported increased apoptosis after delivery of NTR and CB1954 to targeted cells (32) which was presumed to be a result of the DNA alterations incurred. It appears that CD1954 acts more rapidly than other prodrugs such as GCV in the HSV-tk system, with reports of cytolytic activity as early as 4 h after prodrug administration (31,33). One explanation for this comparatively rapid response is that the NTR strategy does not require cells to be in the S phase of growth.

This lack of specificity for dividing cells may prove advantageous in achieving maximal antitumor efficacy; however, it raises a significant safety concern regarding normal surrounding tissues. A critical issue in the future application of this system will be the development of tumor-specific targeting so that normal somatic cells will not be exposed to the cytotoxic effects of the enzyme and prodrug. Another potential drawback to the CB1954/NTR system is that a bystander effect has not been identified to date (32). These 2 limitations may preclude the clinical application and benefit of this system until increased efficiency of gene transfer and tumor-specific targeting is achieved.

E. Cytochrome P450 2B1

The hepatic enzyme cytochrome P450 B1 (CYP2B1) will convert the inert lipophilic prodrug cyclophosphamide (CPA) into an effective anticancer agent (34). CPA is initially converted into 4-hydroxy-cyclophosphamide (4-HCPA), which is naturally unstable and will spontaneously decompose into 2 toxic metabolites, acrolein and phosphoramide mustard (PM). Acrolein will promote covalent links in cellular proteins and PM induces DNA alyklation and results in DNA strand breaks during replication. The importance of acrolein in causing tumor cytotoxicity in vivo has yet to be proven; therefore, the major anticancer metabolite appears to be PM. The cytotoxicity of PM affects both dividing and nondividing cells and so may prove useful for tumors that have low levels of S-phase activity such as the glioblastoma brain tumor (35).

Tumor cells usually express low levels of CYP2B1 but the liver expresses higher levels. Systemic exposure to CPA at concentrations that would prove effective in killing tumor cells will result in high levels of toxic metabolites produced in normal liver tissue. These high levels of toxic metabolites not only injure normal liver tissue, but also are released into the circulation. Systemic toxicity has therefore limited the ability to use these prodrugs alone as cancer chemotherapeutic agents. Gene transfer of the CYP2B1 gene and the resulting upregulated expression of the enzyme, however, will allow for administration of smaller systemic levels of CPA prodrug to maintain tumor cytotoxicity while minimizing systemic toxicity.

One important advantage of this system is that the intermediate metabolite 4-HCPA is lipophilic and so can pass through cellular membranes (36). Diffusion of these metabolites throughout the nontransduced tumor cells results in a strong bystander effect. Despite this apparent augmentation of overall antitumor effects, the free diffusion of 4-HCPA and the fact that the CYP2B1 gene therapy strategy does not require cell division also carries the disadvantage of possible toxicity in surrounding normal tissue.

F. Carboxypeptidase G2

The bacterial enzyme carboxypeptidase G2, (CPG2) has no mammalian homolog and has been shown to activate the pro-

drug 4-[(2-chloroethyl)(2-mesyloxyehtyl0amino]bensoyl-L-glutamic acid (CMDA), which is a derivative of a benzoic acid mustard. The CPG2 enzyme removes the glutamic acid moiety from the CMDA prodrug and releases a toxic benzoic acid mustard (37), which requires no further enzymatic or decomposition process. The benzoic acid mustard is a strong alkylating agent and cross-links DNA, thus imparting toxicity to both dividing and nondividing transduced cells.

The single-step process of converting CMDA to the toxic mustard metabolite offers an advantage over other suicide gene therapy strategies that have intermediate metabolites and multistep conversion processes within a targeted tumor cell. If the cellular enzymes that are responsible for the second phase or multistep activation process become defective or deficient in the tumor cell, a significant resistance to the prodrug could develop (38). However, when the toxic metabolite is released directly from the initial step or prodrug cleavage, there is much less chance of developing resistance to the suicide gene therapy. Also, mustard alkylating agents such as benzoic acid mustard have the advantage that their cytotoxicity is dose related. This important factor further reduces the chances of resistance.

As mentioned with other suicide gene therapy strategies above, this broad killing is beneficial for tumors with significant number of cells in Go at the time of gene transfer and prodrug administration. However, the potential continues to exist for direct toxicity to surrounding normal tissues that are transduced with this suicide gene.

A substantial bystander effect has been documented with the CPG2 strategy in vitro when as few as 3.7% of the tumor cells were expressing CPG2 (39). This is one of the strongest reported bystander effects and serves as an advantage for this strategy.

G. *E. coli* gtp

The *gtp* gene encodes the enzyme xanthine guanine phophoribosyltransferase known as XGPRT. This enzyme converts the xanthine analog, 6-thioxanthine (6-TX) into a weakly toxic purine analog that is subsequently phosphorylated into 6-thioxanthine monophosphate (6-XMP) by the same XGPRT enzyme. 6-XMP is then converted into the very toxic 6-thioguanine monophosphate (6-GMP). This suicide gene therapy has been studied in a retrovirus-mediated gene transfer strategy in sarcoma and glioma tumor cell line and animal model experiments (39a,39b). XGPRT may also prove valuable in adenovirus-mediated gene transfer strategies.

H. *E. coli* Deo

When the *Deo* gene is expressed in target cells, the enyzme purine nucleoside phosphorylase (PNP) is produced. The PNP enzyme is not found in human cells, thus giving this system a level of selectivity and safety with respect to application in human tumors. PNP converts the prodrug 6-methyl purine deoxyriboside (MePdR) into a very toxic metabolite, 6-methyl purine (MeP). MePp is directly toxic to tumor cells and has a strong bystander effect. The main reason for its strong bystander effect is its ability to freely diffuse through cell membranes and travel into surrounding tumor cells. Significant tumor necrosis has been demonstrated when as little as 1 in 100 to 1000 tumor cells have been effectively trasduced with viral vectors carrying the *E. coli Deo* gene. This strong suicide gene therapy effect and corresponding bystander effect has been studied in human colon, glioma, and melanoma cell lines in vitro and ovarian and glioma cell lines in vivo (39c,39d).

IV. THE BYSTANDER EFFECT

Although present to variable degrees for each suicide gene therapy strategy, the bystander effect provides a major advantage for these systems. The advantage of a bystander effect is obvious when considering that present gene transfer approaches provide relatively poor transduction efficiencies of tumors in vivo. Given that only a fraction of any targeted tumor can be effectively transduced, a supplemental antitumor effect that does not require exposure or uptake of the therapeutic gene is critical to the success of suicide gene therapy. As mentioned previously, the bystander effect is a phenomenon whereby neighboring nontransduced tumor cells are killed in conjunction with direct killing of transduced cells (Fig. 1). Multiple theories have been investigated and proposed as an explanation for this effect.

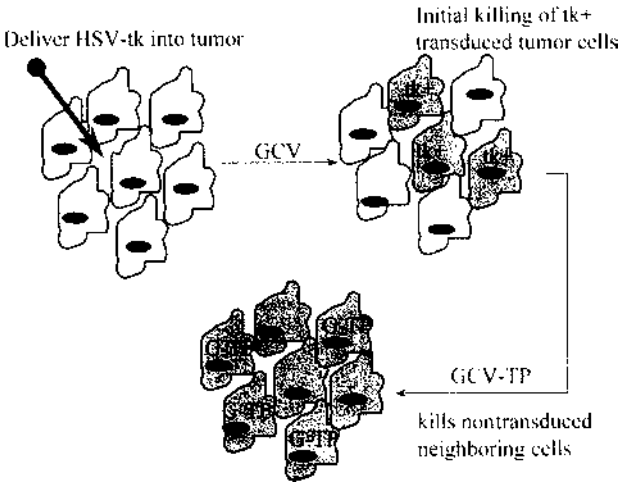

Figure 1 The bystander effect. The HSV-tk gene is delivered to a solid tumor resulting in effective transduction and gene expression in only a percentage of the tumor (shaded cells). Subsequent systemic treatment with ganciclovir (GCV) causes DNA disruption, cell death, and production of toxic metabolites. The toxic metabolites are then passed to surrounding nontransduced cells via gap junctions resulting in cell death.

A. Metabolic Cooperation and Gap Junctions

The most investigated and widely accepted theory to date is that toxic prodrug metabolites are passed between tumor cells through gap junctions. Gap junctions are small hexameric structures (2 nm) in the cell membrane that form part of a communication network between cells. This transfer of toxic metabolites via gap junctional intercelluar communication is founded in the principle of ''metabolic cooperation,'' which was first described by Subak-Sharpe et al. in 1966 (40). Metabolic cooperation is the process whereby low molecular weight molecules (<1000 Daltons) are passed between cells that are in contact. Subsequent to these early studies, it was established that cells with gap junctions were ionically coupled and participated in metabolic cooperation (41). Cells that lacked gap junctions did not participate in this event.

The importance of cell contact in a suicide gene therapy system was identified by Moolten and colleagues who demonstrated bystander killing of nontransduced cells after HSV-tk gene transfer and GCV administration in vitro (10). Although the GCV prodrug can readily and passively diffuse across a cell membrane, the toxic phosphorylated metabolite is not a permeable molecule. However, phosphorylated GCV is approximately 400 Daltons in size and is well within the size limit for metabolic cooperation to occur via gap junctions (42). Subsequent to these early studies with the HSV-tk suicide gene therapy system, Bi et al. introduced the concept that the bystander effect was a result of metabolic cooperation (22). It was demonstrated that labeled phosphorylated GCV was able to enter adjacent contacting tumor cells and resulted in cell death. Gene transfer and expression of a much larger molecule such as β-galactosidase, however, was not transferred to nontransduced contacting tumor cells.

Gap junctional communication is believed to be mediated by a family of proteins called connexins of which 12 genes have been cloned (43). Definitive proof that gap junctional intercelluar communication and connexin activity played a major role in the bystander effect came from the investigations of Mensil et al. (44). In their experiments, HeLa cells were chosen because they exhibit very little gap junctional communication and have no detectable expression of known connexin genes (45). Upon gene delivery of HSV-tk to cultured HeLa cells, only the actual HSV-tk transfected cells were killed despite different levels of cell density or contact. When HeLa cells were transfected with a gene encoding the gap junctional protein connexin 43, both HSV-tk positive and nontransfected surrounding cells in contact were killed. This effect was abrogated when HSV-tk positive and negative HeLa cells were cocultured without cell–cell contact.

B. Transfer of Toxic Metabolites via Apoptotic Vesicles or Direct Transmembrane Diffusion

Another reported mechanism of bystander effect activity is the release of apoptotic vesicles by dying tumor cells after suicide gene therapy. Freeman and colleagues noted that HSV-tk-positive tumor cells exhibited characteristics of apoptosis when dying in culture (21). Microscopic analyses revealed cell shrinkage and detachments as well as chromatin condensation and vesicle formation. Further ultrastructural evaluation using transmission electron microscopy identified features consistent with apoptosis. Apoptotic vesicles released form HSV-tk- and GCV-treated tumor cells could transfer the toxic phosphorylated GCV metabolite or even the HSV-tk gene itself. The mechanism of transfer would involve phagocytoses of these vesicles by surrounding viable tumor cells. This presumed transfer of apoptotic vesicles was demonstrated using a fluorescent tracking dye and fluorescence microscopy and flow cytometry. Nontransfected tumor cells were able to phagocytose the labeled apoptotic vesicles generated from dying HSV-tk transfected cells. Further studies on the importance of this finding in mediating the extent of bystander activity are required before this principle is substantiated and accepted.

Certain toxic metabolites such as 5-FU and 6-methyl purine (MeP) do not require gap junctions or the phagocytoses of apoptotic vesicles in order to enter surrounding tumor cells. 5-FU and MeP are permeable to most cellular membranes and thus can freely diffuse throughout the tumor, resulting in a strong bystander effect that is not restricted by tumor cell-dependent numbers of gap junctions or phagocytotic capabilities.

C. Local Antitumor Immune Responses

Although metabolic cooperation via gap junctions has been consider the major mechanism of the bystander effect, there is a growing interest in the role of the immune system in this phenomenon (Fig. 2). A number of studies have reported the presence of an inflammatory infiltrate in dying or regressing tumors after both HSV-tk and CD gene therapy (46,47). Other investigations have described a lessened response to HSV-tk and GCV therapy for tumors grown in nude mice as compared with immunocompetent animals (23,48). The decreased effect in these athymic and therefore T cell-deficient mice suggests that a T cell-mediated immune response plays some role in tumor regression. In these studies, nude mice and sublethally irradiated mice failed to demonstrate subcutaneous tumor regression when the tumor cell population consisted of 50% HSV-tk-transduced cells. The same experiments in immunocompetent mice, however, did show tumor rejection with the 50% proportion of HSV-tk-transduced cells.

More specific immune and cytokine analyses were subsequently performed using an intraperitoneal tumor model (49). Upon treatment of established tumors with HSV-tk-transduced cells, the peritoneal exudate was analyzed for the presence of various cytokines. Expression of tumor necrosis factor-alpha (TNF-α), interleukin 1-alpha (IL-1α), and IL-6 was identified at 24 h after tumor treatment with HSV-tk-positive cells. After 48 h interferon gamma (INF-γ) was identified and at 96 h, granulocyte-macrophage colony-stimulating factor (GM-CSF) was produced. There appeared to be a defined cascade of cytokine production that was specific to the HSV-

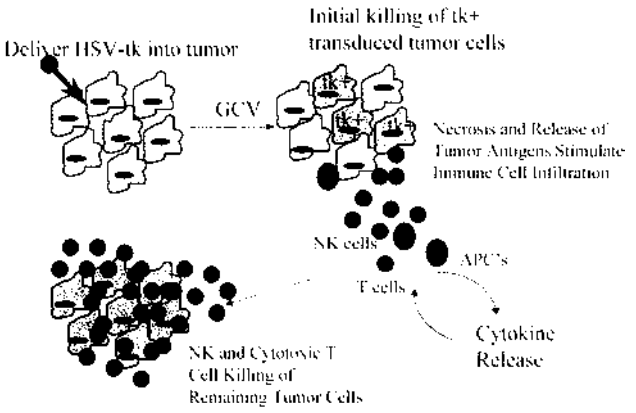

Figure 2 Immune component to the bystander effect. As tumor cells are killed by the suicide gene therapy, there is a release of cellular debris and tumor antigens within the local tumor environment. The cell necrosis and antigen load stimulates recruitment and activation of tumor-specific immune cells. The immune cells attack the local-regional tumor and also initiate a systemic response that may target and kill metastatic disease or prevent recurrence.

tk-treated tumors. Immunohistochemical staining identified immune infiltrates consisting of macrophages and T lymphocytes that were predominantly in and surrounding regressing tumors subsequent to HSV-tk therapy. These representative studies as well as others have established a role for local immune responses in generating killing of both transduced and nontransduced tumor cells. The generation of an antitumor immune response against both transduced and nontransduced tumor cells greatly enhances the power of suicide gene therapy and its associated bystander effect.

D. Systemic Antitumor Immune Responses

There is increasing evidence that not only a direct local immune response but also a systemic immune response is generated from suicide gene therapy. The development of a systemic immunity has been demonstrated for both the HSV-tk and CD strategies. In animal studies with HSV-tk-expressing tumor cells, administration of GCV resulted in both direct tumor cell killing and an ''initial'' protection against a second challenge of wild-type tumor cells (23,50). This effect could not be sustained and eventually tumors grew at the site of second challenge. This initial antitumor immunity was not generated against a second local challenge of syngeneic but heterologous (antigen distinct) tumor cells. Using the same tumor strategy in T cell-deficient nude mice, the antitumor effects and initial systemic immunity against a second-site tumor challenge was greatly reduced. These findings again support the importance of a T cell-mediated tumor-specific immune response. This initial systemic antitumor immunity has been documented in multiple tumor cell types for the HSV-tk strategy, although the extent and period of immune effect has been variable among studies.

Although most of the work centering on immune responses and suicide gene therapy has centered on HSV-tk therapy, the immune component to the bystander effect has also been identified in the CD scheme. Both fibrosarcoma and adenocarcinoma cells that were retrovirally transduced to express CD showed variable levels of resistance to second wild-type tumor challenges subsequent to systemic administration of the prodrug 5-FC (51). Second tumor challenges with antigen distinct tumor lines from the same murine background, however, displayed normal tumor growth, indicating a tumor specificity to the immune response. These findings parallel the results noted in the HSV-tk system.

In the previous investigations, the fibrosarcoma and adenocarcinoma cell lines were generated via carcinogen induction. Subsequent studies have confirmed both a local and systemic protective immune responses after CD and 5-FC therapy in a spontaneously occurring mammary carcinoma (49). Antibody depletion against specific lymphocytes was performed in the animal tumor model system to further define specific components to these immune responses. Upon antibody depletion of CD8-positive T lymphocytes in vivo, a significantly decreased level of local tumor regression and even increased tumor growth was noted in CD-expressing tumors that were treated with 5-FC. Antibody depletion of granulocytes also appeared to limit the antitumor responses. Deletion of CD4 T lymphocytes had no significant effect on local tumor regression from the CD and 5-FC treatment. Interestingly, depletion of CD4-positive T lymphocytes dramatically reduced or in some cases eliminated the systemic immunity against a second local challenge of wild-type tumor. Depletion of CD8 T lymphocytes also appeared to limit the systemic antitumor bystander effects of a second challenge of wild-type tumor. Overall, these studies support the ability of suicide gene therapy to generate some level of systemic immunity. The systemic protection, however, was present at variable levels and only short-term systemic immunity was evaluated.

Although systemic immune responses can be generated against second tumor challenges after suicide gene therapy, could it be possible to induce regression of existing tumor metastases after treatment of the primary tumor? Original murine studies in multiple tumor types evaluating responses against an established subcutaneous wild-type tumor at a second site were not encouraging. Tumor killing and regression identified in subcutaneous flank tumors that expressed CD or HSV-tk did not induce a significant effect on wild-type (nontransduced) tumors growing on the opposite flank (21,26,51). This apparent lack of effect on the presence of established tumors at a distant site suggested that the immune bystander effect was not going to be effective against gross metastases present at the time of primary tumor therapy.

Further studies involving orthotopic metastases, however, have defined some role of this immune bystander effect in the treatment of metastatic disease. Consalvo et al. demonstrated regression of lung metastases in 20% of mice whose primary CD-expressing subcutaneous tumors regressed after 5-FC ad-

ministration (47). Up to 90% regression of HSV-tk-negative liver metastases was also described after elimination of HSV-tk-expressing tumors growing intraperitoneal in a murine colon adenocarcinoma model (52). Immunohistochemical studies confirmed the presence of increased inflammatory infiltrates in these regressing metastases as compared with controls. These representative studies support the concept that a systemic antitumor immunity or bystander effect is capable of generating variable levels of tumor regression in distant metastases.

The issue has not been resolved as to why established second site wild-type tumors are not affected by suicide gene therapy directed at the primary tumor but metastatic lung and liver tumors show some regression. Excluding possibilities such as natural antigenicity of various tumor types and inherent differences among animal tumor models, there are a few hypotheses to explain this somewhat contradictory finding. The first hypothesis is that anatomic location of second site or metastatic disease is an important issue in generating effective immune-mediated regression after primary tumor therapy. The lung and liver have an increased number of cells from the reticuloendothelial system as compared with the subcutaneous tissues. The increased reticuloendothelial cells surrounding the metastases may facilitate or enhance antitumor immune activity generated from the initial primary tumor treatment. Also, this environment within the lung or liver may promote increased or more efficient antigen presentation. The second hypothesis may be related to gross tumor burden. Although in multiple animal models a second site established tumor has not regressed after primary tumor treatment with suicide genes, an initial protection against repeat tumor challenges has been well documented. The second hypothesis is based on this finding. It is possible that the systemic immune response generated from the initial tumor treatment is not strong enough to manage the gross tumor burden of an established tumor but can handle the smaller tumorigenic doses given for a second challenge. Further experiments to evaluate this consideration of tumor burden or rate of cell division within the metastatic lung and liver models may help to answer this question.

V. COMBINATION SUICIDE GENE AND CYTOKINE GENE THERAPY

Based on the initial immune responses generated after suicide gene therapy, there has been a recent interest in combining this strategy with cytokine gene therapy. Cyotokine gene therapy has arisen because of the growing opinion that tumor-specific antigens are expressed in many if not all human tumors, but the immune system fails to generate an adequate immune response against these antigens (53). Designing or discovering a means to augment baseline immune activity or generate tumor-specific responses would greatly enhance any cancer treatment strategy. Systemic infusions of cytokines has demonstrated tumor regression in both animal tumor models and some human clinical trials; however, the severe toxicity associated with the high concentrations required to achieve antitumor efficacy significantly limits this strategy (54–56). Cytokine gene therapy may circumvent the toxicity of systemic infusions and provide both larger and sustained local concentrations of cytokine within the tumor environment. This augmentation of the immune response with cytokine production may also be synergistically enhanced by the addition of suicide gene therapy.

Upon treatment of a tumor with suicide gene, direct cytotoxicity and tumor cell death occurs. This tumor killing releases large amounts of tumor antigens as well as other cellular proteins and debris. As has been demonstrated previously, this results in variable levels of immune activity in and of itself. The suicide gene therapy not only reduces the gross tumor burden, but also primes the local tumor immune environment for the beneficial effects of cytokine production. In addition, the actual suicide gene proteins themselves are believed to be immunogenic and may act as superantigens for both nonspecific and tumor-specific lymphocyte activation. By introducing high levels of local cytokine production using combined gene transfer techniques, this overall antitumor immune response may be greatly magnified.

Pioneering this area has been Chen and Woo et al. who investigated the effects of combination HSV-tk and IL-2 gene therapy for colorectal cancer liver metastases (57). Upon injecting the liver metastases with a combination of HSV-tk and IL-2, a significantly increased level of tumor regression was noted as compared with either HSV-tk or IL-2 alone. A long-term survival benefit was not seen. The combination treatment did generate an initial systemic immunity against a second challenge of wild-type tumor at a distant site. This response was tumor specific, but was not sustained. Immune studies revealed a predominantly CD8-positive T lymphocyte response. Interestingly, the treatment of the liver metastases with HSV-tk alone did not demonstrate a significant effect with respect to protection against a second- site tumor challenge. When applied to a head and neck orthotopic squamous cell carcinoma model, the combination of HSV-tk and IL-2 gene therapy demonstrated both a synergistic antitumor response and increased survival as compared with single gene therapy (58). The combination of HSV-tk and IL-2 in this head and neck cancer model also generated an immediate systemic immunity and protection against a second-site challenge of wild-type tumor (53). Immunohistochemical analyses local tumor environments revealed a predominance of CD8-positive T cell infiltration, but also CD4-positive T cells were identified.

Further investigations with the combination of HSV-tk, IL-2, and GM-CSF have demonstrated both enhanced tumor regression and animal survival. Also, this triple combination has shown that systemic immunity can be sustained as long-term protection against second wild-type tumor challenges could be generated (59). It is hypothesized that the addition of GM-CSF not only stimulates antigen presentation cells within the local tumor environment, but induces a long-term antitumor memory that is mediated by CD4-positive T cells (60) However, not all tumors respond to triple combination therapy, and there may inherent factors within the tumor type

or even the anatomical region of the body that influences such responses to the addition of GM-CSF (60a).

Given the low in vivo gene transfer efficiency of many viral vectors, certain strategies have been studied that may circumvent these limitations. VP22 is a protein normally produced by the HSV-1 virus and has a unique capability of achieving intercellular spread (60b). The VP22 protein is actively exported from the cytoplasm of cells producing the protein, and then it is subsequently transported across neighboring cell membranes where it is delivered to the cell nucleus. VP-22 fusion proteins and genes fused with VP22 (i.e., VP22-tk, VP22-p53, CD-VP22) are able to retain these intercellular transport properties (60c,60d). Wybranietz et al. demonstrated a significant benefit of adenovirus-mediated CD-VP22 fusion gene delivery vs. adenovirus CD suicide gene therapy alone in multiple tumor cell lines. Gene therapy strategies employing VP22 fusion proteins present new horizons for improving the efficiency and overall efficacy of suicide gene therapy strategies.

Another possibility for improved suicide gene therapy is to combine two powerful suicide genes into one delivery vehicle or viral vector. Various studies have investigated the potential for adenovirus-mediated HSV-Tk and CD double suicide gene therapy in both preclinical and clinical investigations (60e). Moriuchi et al. did not identify a benefit of HSV-TK and CD double suicide gene therapy with respect to killing of glioma tumor cells (insert reference Moriuchi S). A replication-defective adenovirus vector was used in these experiments. A number of investigators, however, have demonstrated in preclinical studies the benefit of double suicide gene therapy strategies in combination with radiation for various tumors cell lines (60f,60g). The application of double suicide gene therapy in human clinical trials are discussed at the end of the chapter.

VI. CLASSIC METHODS FOR SUICIDE GENE TRANSFER

There are 2 classic areas of investigation regarding the application of suicide genes for the treatment of solid malignancies. The first area focuses on the development of suitable vehicles for introducing suicide genes into targeted tumor cells. The second area is the discovery and development of new suicide genes or gene combinations that will provide the greatest antitumor response while maintaining margins of safety. Regarding the area of gene transfer, 2 general classes of vehicles and transfer methods can be distinguished. *DNA-mediated gene transfer* involves the administration of DNA in the form of a circular double-stranded plasmid directly to the tumor. The vehicles for introducing therapeutic DNA into tumor cells are many. They include both mechanical methods and a more promising method that uses a wide variety of lipid or polymer formulations that are designed to maximize gene uptake and expression within the tumor cells. *Viral-mediated gene transfer* involves packaging a therapeutic gene into a replication-defective virus particle and using the natural process of viral infection to introduce the gene. The purpose of viral-mediated gene transfer is to exploit the efficient and often complex mechanisms that viruses have evolved to introduce their viral genes into human cells during infections. DNA-mediated transfer, while being less efficient than viral transfer, does not carry the risks of wild-type virus contamination and other known viral-associated toxicity. Furthermore, treatment limiting immune responses are more prevalent with the use of viral vectors, in general, than DNA transfer systems that may allow repeat gene delivery over a long period of time.

A. DNA-mediated Gene Transfer

DNA-mediated gene transfer is commonly called transfection. The therapeutic gene is typically contained within a circular molecule of DNA (plasmid), which contains various additional genetic elements required to achieve expression of the gene product. Important components to these plasmids are special promoters and enhancers that direct gene expression. There may also be other specific elements that have been engineered to direct the processing and persistence of genetic material within the cell.

There are many methods for delivering plasmid DNA into tumor cells, but only a few are clinically applicable at present. A classic means of introducing DNA into cells in vitro is through electroporation, where cultured cells are exposed to DNA in the presence of a strong electrical pulse (61). The electrical pulse creates pores in the tumor cell membrane, allowing entry of DNA into the cell. A recent technical advance using a similar principle has been the development of the ''gene gun.'' This mechanical device uses electrical currents and magnetic properties to project gold-coated DNA vectors into the tumor cells (62). At present, it is limited to cases where direct visualization and broad access to the tumor is possible.

It is possible to effectively introduce genes into muscle (63) or thyroid (64) simply by directly injecting DNA in saline solution into these tissues, whereupon the process of endocytosis enables cellular uptake. The use of saline as a gene transfer vehicle, however, is extremely inefficient for solid tumors and generally not applicable for cancer suicide gene therapy. The state of the art technology for DNA-mediated gene transfer into solid tumors is the use of liposomes or cationic lipid complexes. These lipid formulations are specifically designed to enhance DNA uptake and may be modified to provide higher levels of gene delivery to different histologic tumor cell types. (65). The use of lipid or liposome formulation gene transfer is becoming a major focus for clinically applied cancer gene therapy.

There are two limiting factors with respect to DNA-mediated gene transfer in general. First, the overall efficiency of gene transfer is low with in vivo expression levels of 1% to 3% at best even with cationic lipid or polymer formulations. Although suicide gene therapy strategies do not require gene transfer into all or even a majority of tumor cells for effective

killing, there is a correlation between levels of gene expression and antitumor effects. This correlation affects both the direct cytotoxicity and the bystander effect. In general, levels of transduction lower than 10% of the tumor population in vivo have very limited effects on either tumor regression or delay in tumor progression. The second limiting factor for DNA-mediated gene transfer is that the therapeutic gene usually resides or functions transiently in the targeted cell, regardless of the formulation. Subsequent to DNA-mediated gene transfer the therapeutic genes are degraded and eliminated from the tumor cell over a short period of time. This factor may be less important for suicide gene therapy as the targeted tumor cells are generally killed within days to a week after initiating prodrug administration. It is also possible to perform repeat dosing with DNA-mediated gene transfer. Sugaya et al. reported antitumor effects of 10 serial DNA-mediated HSV-tk treatments for colon adenocarcinoma with each injection providing only a maximal of 1% to 2% in vivo transduction efficiency (66).

Permanent incorporation of genes into cells occurs rarely after DNA-mediated gene transfer in cultured cells ($<1:10^5$ cells); however, this phenomenon has not been observed in vivo. Although the transient expression of the therapeutic gene may be a disadvantage in certain treatment schemes, there are also may advantages to this property. Because DNA vectors do no integrate into a host cells chromosomes (i.e., in the case of normal tissue surrounding a tumor cell), they do no carry the risk of permanent alteration of a normal tissues genome. The transient nature may also limit toxicity associated with any gene therapy system. The lack of significant immune responses against the DNA vector also allows repetitive administration of the therapeutic gene into persistent or recurrent tumors. The ability to perform repeat treatments may overcome the potential limiting aspect of transient gene expression.

B. Retrovirus-mediated Gene Transfer

The majority of suicide gene therapy preclinical and human trial research to date has centered on the use of viruses as gene delivery vehicles or vectors. The original prototypes for viral-mediated gene transfer are retroviral vectors derived from the Moloney murine leukemia virus (67,68). Retroviral vectors were chosen as vehicles because of several useful properties. First, ''defective'' virus particles can be constructed that contain therapeutic genes and are capable of infecting cells, but which contain no viral genes and express no pathogenic viral gene products. Second, retroviral vectors are capable of integrating the therapeutic genes they carry into the chromosomes of the target cell resulting in long-term gene expression. Third, modifications can be made in retroviral vectors and in the cell lines producing vectors, which result in enhanced safety features.

There are certain limitations to consider regarding the application of retroviruses in gene therapy for cancer. Retroviruses will only integrate into actively dividing cells. Because most tumors have heterogenous populations of dividing and nondividing cells, many tumor cells that are exposed to the retrovirus vehicle may never take up the therapeutic gene. Moreover, the currently achievable viral titers (10^7) are relatively low and the overall efficiency of retroviral infection of tumors in vivo is low. Because of this low level of in vivo transduction, the retrovirus must be delivered to the tumor via its murine or human packaging cell line. Also, serum complement can inactivate retroviruses that are produced from packaging cell lines that are not of human origin (69). Another important limitation is the variable receptivity of host cells to retroviral infection. Finally, under large-scale retrovirus production there is a risk of producing replication-competent viruses. These last 2 limitations are being addressed through modifications of the packaging cell lines (70,71).

C. Adenovirus-mediated Gene Transfer

A recent focus of gene therapy has been the development of adenovirus vectors as powerful and effective vehicles for gene transfer. Adenoviral vectors differ from retroviral vectors in that they do not integrate their genes into the target cell's chromosome. Compared with retroviral vectors, adenoviral vectors can be produced at much higher titers (10^{11} or greater) and can efficiently transduce a wide variety of both dividing and nondividing cells in vitro and in vivo (72). Hematopoietic cells are the only cells that have demonstrated significant resistance to adenovirus infection thus far. Effective therapeutic gene expression is transient after adenovirus gene transfer and typically lasts for 1 to several weeks. There are both advantages and disadvantages to this last characteristic. Although the safety of adenoviral vectors for gene therapy has not been studied as extensively as retroviral vectors, there is considerable experience with the use of attenuated adenovirus in animal models and in human subjects indicating a high margin of safety (73,74).

The disadvantages with the adenovirus system must be considered when designing and applying gene therapy strategies. Although the adenovirus vector is replication defective, it will express certain viral gene products or antigens that are capable of inducing an inflammatory response and subsequent lysis of the transduced cell (75). This inflammatory response could result in injury to normal surrounding tissues or even distant tissues that have been infected with the adenovirus vector. The generation of antibodies against the adenovirus vector may severely limit repeat adenovirus gene therapy treatments. Also, the transient level of gene expression may limit the desired therapeutic effect. As has been mentioned previously, transient gene expression may not be a significant disadvantage with a suicide gene therapy strategy. The potential disadvantages of transient gene expression and limiting inflammatory responses have been addressed with further manipulation of the adenovirus genetic backbone. New ''second-generation'' vectors, which remain for months in the target cell, express less viral proteins, and have greatly reduced inflammatory responses, are now available and under continued modification and development (76,77).

D. Other Viral Vectors

There exist other viruses that exhibit properties that may be useful to exploit for suicide gene therapy or other gene therapy strategies. The adeno-associated virus can provide a completely defective vector with the majority of the viral genome replaced by the therapeutic gene (78). As a result, this virus appears to generate less immune responses and has a longer period of therapeutic gene expression than the classic adenovirus vector. Unlike retrovirus, the adeno-associated virus integrates in a specific location on chromosome 19, thereby reducing the risk associated with randomly integrating vectors. Although a theoretical advantage, recombinant adeno-associated virus appears to have lost this predictable integration advantage (79). Another disadvantage of the adeno-associated virus is the requirement for wild-type ''helper virus'' in the vector production process. The ''safe'' recombinant vector must then be purified from the wild-type helper virus prior to amplification and in vivo application. Also, the viral titers are low for adeno-associated virus (10^4), in part because of the inefficient production process. Further investigation is required to define the role and safety of adeno-associated virus in clinical application.

The herpes simplex virus is capable of infecting cells and persisting indefinitely in a latent state. Vectors using the herpes virus have been constructed which are replication defective and capable of expressing recombinant genes for prolonged periods of time in animal models (80). These viruses are not completely defective and continue to express many viral proteins that can be cytopathic, a property that severely limits the herpes virus for present gene therapy applications.

Other viruses, including the human papilloma virus, vaccinia virus, avipox virus, and baclovirus, are under investigation for gene therapy application. The improvements and continued refinements of presently available viral vectors as well as the development of new vectors should greatly expand the efficacy and applicability of suicide gene therapy.

VII. GENERAL STRATEGIES FOR SUICIDE GENE THERAPY

Two basic strategies exist for cancer gene therapy. The first conceived strategy is ''ex vivo'' gene therapy in which a tumor or fibrous tissue biopsy is taken from a cancer patient whereupon individual tumor cells or fibroblasts are isolated and grown in vitro (Fig. 3). Therapeutic genes are then inserted into these cells typically using retroviral vector infection in tissue culture. The cells are subsequently irradiated and then reimplanted into the original tumor site or distant to the tumor site by autologous transplantation. The level of irradiation is controlled so as not to immediately kill the cells but to prevent growth and allow only a short period of survival after reimplantation. This strategy has been more commonly applied to classic cancer vaccine and cytokine gene therapy strategies. Although this approach is feasible with suicide gene therapy, the predominant tumor response would come from a metabolic cooperation or immune bystander effect subsequent to prodrug

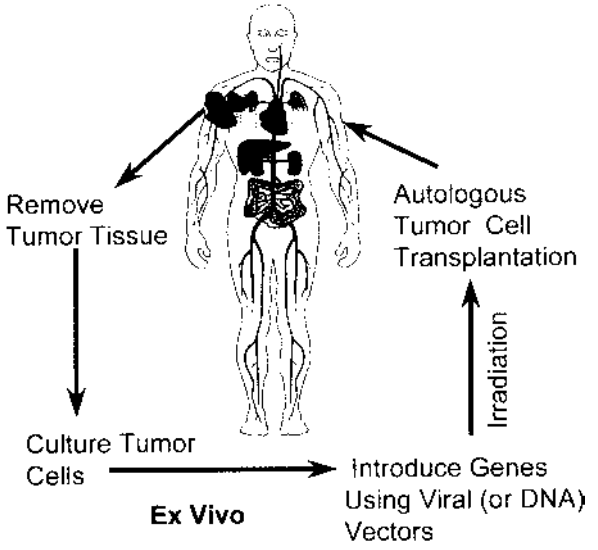

Figure 3 Diagram of the ''ex vivo'' strategy for suicide gene therapy.

administration and killing of the reimplanted transduced tumor cells. The inefficiency of such a system in general supports the predominant research and clinical focus on in vivo approaches for suicide gene therapy.

The second is basic strategy for cancer gene therapy is ''in vivo'' gene therapy where DNA, viral vectors alone, or packaging cell lines producing viral vectors are administered directly to a cancer patient (Fig. 4). The most common route of delivery is via direct injection of the delivery vehicle and gene or the packaging cell line into the tumor. Systemic injection is possible, but with present technology, results in limited exposure of the tumors to the vehicle. Tumors within tissues that have a large amount of blood flow or act as filters such as the lung or liver may prove more amenable to systemic delivery. As gene transfer technology continues to advance, the development of vehicles with tumor- or tissue-specific receptor uptake or promotor activity may allow for systemic administration of the DNA or viral vector carrying the suicide gene.

A. REPLICATION-COMPETENT VIRAL THERAPY: A FORM OF ''SUICIDE THERAPY''

Until very recently, viral vectors used in all gene therapy strategies were replication incompetent. Although this design was intended to introduce safety measures, it does not take advantage of the powerful ability of viruses to infect target cells and replicate and release viral particles, thereby killing target cells and spreading to surrounding target cells. Continued research and investigation in the fields of molecular biology and the genetics of cancer have led to a greater under-

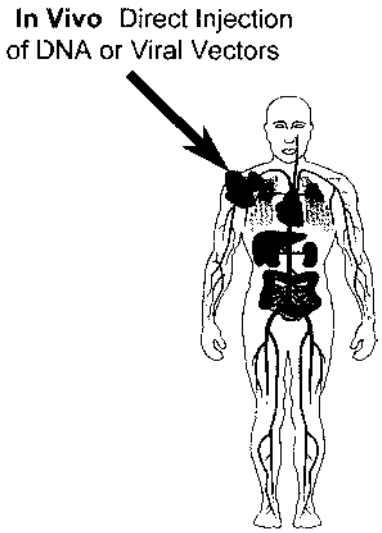

Figure 4 Diagram of the ''in vivo'' strategy for suicide gene therapy.

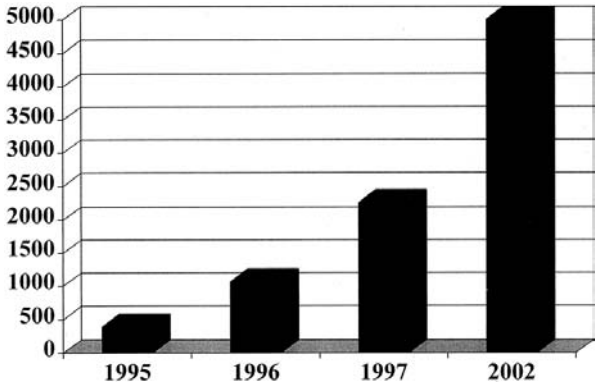

Figure 5 Trend for patient enrollment for human gene therapy trials.

standing of the principles of viral replication and the genetics of carcinogenesis. This greater understanding has allowed the development of replication-selective oncolytic viruses for use as novel anticancer therapies.

The first replication-selective viral vector to move from preclinical studies to human cancer clinical trials was the Onyx-015 adenovirus, also known as dl1520 (81,82). The key alteration that made this adenovirus replication selective was the deletion of the gene that codes for the p53-binding protein, E1B-55kDa. Typically, the adenovirus achieves replication in part through a process by which the E1B-55kDa protein binds a host cells p53, thereby allowing the cell to enter the S-phase of cell-cycle activity. The dl1520 adenovirus with its deletion of the E1B gene will not express the E1B-55kDa p53-binding protein upon infection of a target cell. The lack of E1B-55kDa expression will inhibit viral replication. However, in target cells that lack normal p53 expression, the dl1520 virus will maintain its ability to replicate, lyse a target cell, and spread to nearby cells. Because the majority of cancers have a loss of normal p53 function, cancer cells are the ideal target for a E1B-deleted replication-selective adenovirus therapy. There are many other evolving strategies for oncolytic viral vector therapy, and it is not the purpose of this chapter to discuss the present state of this novel therapy in detail. However, it is important to mention replication-selective oncolytic viral therapy because it may be thought of as a type of gene-dependent suicide therapy.

VIII. CLINICAL TRIAL SUMMARY FOR SUICIDE GENE THERAPY STRATEGIES

As of July 2002, there were 636 human gene therapy clinical trials that were either initiated or completed worldwide with an estimated patient enrollment of 5000 (83,84). A summary of the progression of patient enrollment is depicted in Fig. 5, and a breakdown of the trials by diseases in Fig. 6. Of these human clinical trials, approximately 505 have been or are being conducted in the United States with the next highest number of trials at 43 for the United Kingdom. Of these total human clinical trials, 403 are directed at cancer and 55 of these cancer trials are evaluating suicide gene therapy.

With respect to the vectors being used, Fig. 7 showns the distribution to date. The retrovirus remains the most commonly studied vector at approximately 34% of all trials. The adenovirus has advanced to a close second at 27% of all trials. The largest increase over the past 5 years has been in the strategy of nonviral gene delivery with approximately 23% of all trials focusing on this method. The distribution within nonviral trials is evenly split between lipid-mediated and naked plasmid-mediated gene transfer.

The ongoing or completed clinical trials for suicide gene therapy and cancer overall have provided a few important points for human gene therapy investigation. The first point is that retroviral, adenoviral, and DNA-mediated vectors are at present overall safe vehicles for gene transfer. Except for the

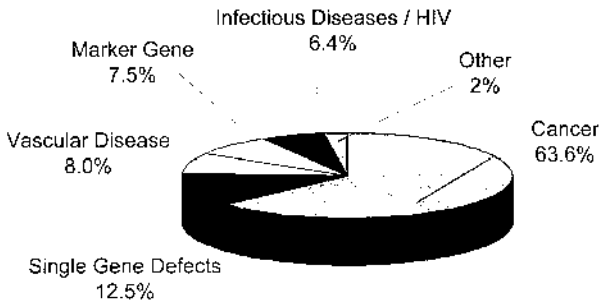

Figure 6 Distribution of human clinical trials by disease state.

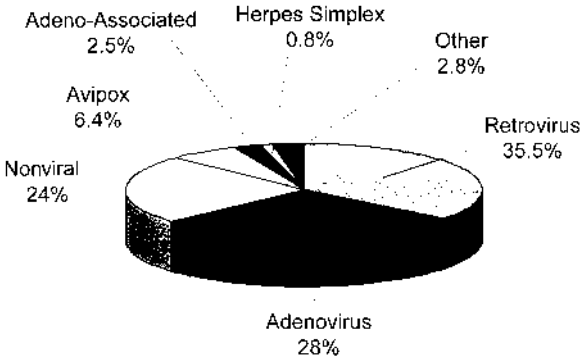

Figure 7 Distribution of vectors used in human clinical trials.

unfortunate death of a young man in 1999 who was enrolled in a gene therapy trial for congenital liver disease, there have been rare and limited side effects with the collection of vectors and genes under investigation (84,85). There have been no significant adverse events with nonviral or DNA-mediated gene transfer in human trials to date. The second important point is that gene transfer and expression in human cancer cells in vivo is possible with both viral and nonviral strategies. The last point is that tumor responses or reports of regression must be interpreted cautiously because the majority of trials remain in phase I or phase I/II and have limited controls for measuring outcomes. Fig. 8 depicts the breakdown of trials with respect to the phase.

At this early stage of human clinical trial investigation, the studies have focused on patients with advanced or incurable cancer. Although this patient population is a standard choice for establishing the safety of novel therapies, the greatest chance of eventual success with presently available suicide gene therapy strategies may be found in patients with less advanced disease. Another important potential for suicide gene therapy is in combination with immune therapies or standard therapy such as with surgery or radiation. As advancements in both preclinical development and clinical application continue, suicide gene therapy should achieve a role in the treatment of cancer.

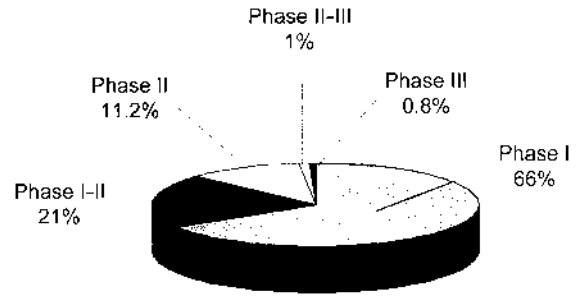

Figure 8 Distribution of human gene therapy trials by phase.

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21

Selectable Markers for Gene Therapy

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I. INTRODUCTION

A. The Use and Choice of Selectable Markers

One of the major problems with current approaches to gene therapy is the instability of expression of genes transferred into recipient cells. Although in theory, homologous recombination or use of artificial chromosomes can stabilize sequences with wild-type regulatory regions, such approaches to gene therapy are not yet feasible and may not be efficient for some time to come. In most high efficiency DNA transfer in current use in intact organisms, selectable markers must be used to maintain transferred sequences; in the absence of selection the transferred DNAs or their expression is rapidly lost.

There are several different selectable markers that might be used for in vivo selection, including genes whose expression has been associated with resistance of cancers to anticancer drugs. Examples include: (a) methotrexate resistance due to mutant dihydrofolate reductase [DHFR] (1); (b) alkylating agent resistance due to expression of methylguanine methyltransferase [MGMT] (2); and (c) the expression of the multidrug transporting proteins P-glycoprotein (P-gp, the product of the *MDR*1 gene) (3) and MRP (multidrug resistance associated protein) (4). In this chapter, we will detail our experience with the *MDR*1 gene.

The resistance of many cancers to anticancer drugs is due, in many cases, to the overexpression of several different ATP-dependent transporters (ABC transporters), including the human multidrug resistance gene *MDR*1 (ABC B1) (3,5,6), MRP1 (ABC C1), the multidrug resistance-associated protein (7) and other MRP family members (8), and *MXR* (ABC G2) (9). *MDR*1 encodes the multidrug transporter, or P-glycoprotein (P-gp). P-gp is a 12 transmembrane domain glycoprotein composed of 2 homologous halves, each containing 6 transmembrane (TM) domains and one ATP binding/utilization site. P-gp recognizes a large number of structurally unrelated hydrophobic and amphipathic molecules, including many chemotherapeutic agents, and removes them from the cell via an ATP-dependent transport process (see Fig. 1).

MDR1 has many obvious advantages for use as a selectable marker in gene therapy. It is a cell surface protein that can be easily detected by FACS or immunohistochemistry. Cells expressing P-gp on their surfaces can be enriched using cell sorting or magnetic bead panning technologies. The very broad range of cytotoxic substrates recognized by P-gp makes it a pharmacologically flexible system, allowing the investigator to choose among many different selection regimens with differential toxicity for different tissues and different pharmacokinetic properties. Furthermore, as will be discussed in detail in this chapter, P-gp can be mutationally modified to increase resistance to specific substrates and alter inhibitor sensitivity. Hematopoietic cells initially appeared to tolerate relatively high levels of P-gp expression without major effects on differentiated function (10).

B. Lessons from Transgenic and Knockout Mice

Two lines of evidence support the concept of using *MDR*1 as a selectable marker in human gene therapy. Transgenic mice

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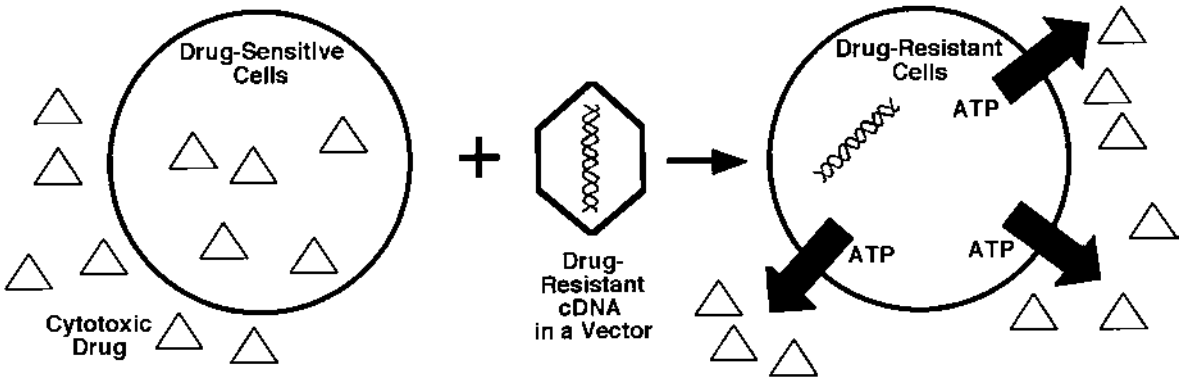

Figure 1 Multidrug transporters such as MDR1, MRP1, and MXR confer resistance on transduced cells. The triangles are cytotoxic drugs; the large, bold arrows are transporters.

expressing the *MDR*1 gene in their bone marrow are resistant to the cytotoxic effects of many different anticancer drugs (10–12). *MDR*1 transgenic bone marrow can be transplanted into drug sensitive mice, and the transplanted marrow is resistant to cytotoxic drugs (13). Mice transplanted with bone marrow transduced with the human *MDR*1 cDNA and exposed to taxol show specific enrichment of the *MDR*1-transduced cells (14–16), and this transduced marrow can be serially transplanted and remains drug resistant (16). Recently, this ability to select transduced bone marrow with taxol has been demonstrated in a canine bone marrow transplantation model (17).

The mouse *mdr*1a and *mdr*1b genes have been insertionally inactivated in mice (18–21). These animals, although otherwise normal, are hypersensitive to cytotoxic substrates of P-gp. This hypersensitivity is due in part to the abrogation of the *mdr*1a-based blood brain barrier (22), and to enhanced absorption and decreased excretion of *mdr*1 substrates (23). These studies demonstrate the critical role that P-gp plays in drug distribution and pharmacokinetics, and argue that specific targeting of P-gp to tissues that do not ordinarily express it (as in gene therapy), will protect such tissues from cytotoxic *mdr*1 substrates.

II. SELECTABLE MARKERS IN HEMATOPOIETIC SYSTEMS AND IN THE SKIN

As noted above, studies on mice transgenic for human *MDR*1 established that constitutive overexpression of this gene protects animals from antineoplastic agents. Drugs could be administered safely at dose-levels several-fold higher than to mice of the respective background strains (10,11). To demonstrate the specificity of this protection, verapamil, an inhibitor of P-glycoprotein, was coadministered, resulting in reversal of drug resistance (12). Similarly, mice transgenic for a mutated dihydrofolate reductase (DHFR) or an O^6-methylguanine DNA methyltransferase cDNA were protected from methotrexate or 1,3-bis (2-chloroethyl) nitrosourea (BCNU) toxicity, respectively (26–24).

Upon overexpression in target cells, drug resistance genes may also protect them from environmental toxins such as carcinogens in addition to amelioration of anticancer chemotherapy (27). For instance, transfer of O^6-methylguanine methyltransferase increases repair of DNA damage in sensitive cells. In vitro and in vivo studies confirmed this aspect of the function of drug resistance genes (28–29). Liu et al. (30) showed that rapid repair of O^6-methylguanine-DNA adducts in transgenic mice protected them from N-methyl-nitrosourea-induced thymic lymphomas. This protection from carcinogens can be targeted to other organs like liver or skin by suitable promoter systems (31,32).

Chemoprotection exerted by overexpression of chemoresistance genes in hematopoietic organs of transgenic animals could be transferred by transplantation of bone marrow to normal recipients (13,33). These experiments provided a basis for gene therapy approaches with drug resistance genes. Hence, drug resistance genes that were initially studied because of their association with failure of anticancer chemotherapy are expected to serve as useful tools for gene therapy of cancer by protecting patients from the toxic side effects of chemotherapy. Protection of chemosensitive cells from toxic compounds may be particularly helpful in the case of the hematopoietic system because most cells in blood and bone marrow are highly susceptible to antineoplastic compounds. $CD34^+$ hematopoietic progenitor cells do not express glutathione-S-transferases (34), and only very low levels of the endogenous *MDR*1 gene are expressed in myeloid and erythroid progenitor cells (35,36). These low expression levels are not capable of providing protection from the cytotoxicity of anticancer drugs. Conversely, the high susceptibility of normal hematopoietic cells to cytotoxic agents allows selection strategies exploiting drug resistance genes if sufficient levels of resistance can be conferred.

A variety of different genes have been used to confer drug resistance on bone marrow cells (see Table 1). Retroviral transduction with a full-length *MDR*1 cDNA promoted by Long-Terminal Repeats (LTRs) of Harvey sarcoma virus protected normal, clonogenic hematopoietic precursors or erythroleukemia cells from anticancer drugs (37,38). Transduced cells were found to be resistant to multiple drugs including taxol, colchicine, and daunomycin. Murine hematopoietic stem cells originating from fetal liver (39), peripheral blood following mobilization with the use of growth factors (40), or from bone marrow (41), were efficiently transduced with retroviral *MDR*1 vectors. In the latter study, it was shown that transplantation of transduced hematopoietic stem cells results in efficient expression of functional human P-glycoprotein in recipient mice. In spite of generally lower transduction frequencies, $CD34^+$ human progenitor cells could also be transduced with retroviruses conveying the multidrug resistance gene (42,43). Similarly, vectors containing MRP1 (44) or mutated DHFR cDNAs are highly efficient in rendering bone marrow cells resistant to methotrexate or trimetrexate, respectively (45,46).

Pluripotent human hematopoietic stem cells or early progenitors, respectively, are difficult to transduce with amphotropic retroviruses (47). Fruehauf et al. (48) targeted immature, cobblestone area-forming progenitor cells. However, in this study significant vincristine resistance was achieved only in a small minority of the immature cell population. This might be due to endogenously high *MDR*1 expression in hematopoietic stem cells (49), which can make it difficult to analyze the function of the transgene. DHFR might be a better marker for selection at the level of long-term culture-initiating cells (50).

Transplantation of *MDR*1-transduced murine bone marrow cells into W/W^v mice (14) or lethally irradiated normal syngeneic mice (15) resulted in significant gene expression in the bone marrow of recipient animals. Both investigators detected elevated levels of *MDR*1 expression after treatment of recipient mice with taxol, favoring the idea of a selective advantage in vivo of hematopoietic cells overexpressing the *MDR*1 transgene. This observation was in marked contrast to previous studies with selectable markers such as genes conferring resistance to neomycin, puromycin, or hygromycin. Because of their pharmacology or pharmacokinetics such compounds cannot be used for selection in vivo.

Further support for the potential usefulness of drug resistance genes for selection in vivo was provided by experiments in which *MDR*1-transduced bone marrow was first transplanted into recipient mice (16). After taxol treatment of recipient mice, their bone marrow was then retransplanted into a second generation of recipient mice. In several cycles of retransplantation and taxol treatment of recipient animals, increasingly high levels of drug resistance were generated in vivo. Mice of the fifth and sixth generation survived doses of taxol that were lethal for mice that had not undergone bone marrow transplantation.

Bunting et al. (51) reported that transduction of murine bone marrow cells with a pHaMDR1 retroviral vector enables *ex vivo* stem cell expansion, which might help account for the ability of transduced cells to survive multiple cycles of transplantation. However, the biological safety of expansion of transduced stem cells is currently under scrutiny. When *MDR*1-transduced progenitor cells are expanded with growth factors for extended periods (up to 12 days), uncontrolled proliferation occurs, as has been observed in one study (51). The authors concluded that the finding was related to the *MDR*1 transgene because the development of a myeloproliferative syndrome was not observed following transfer of DHFR. Other groups have not observed this adverse effect after *MDR*1 transfer to murine hematopoietic cells. More importantly, comparable studies in nonhuman primates did not reveal perturbations of myelopoiesis (52). It appears possible that these differences are related to the recent finding that the integration sites of retroviral vectors reveal predilections for certain chromosomes and are not randomly distributed in the genome of bone marrow repopulating cells (53).

These concerns have prompted recent investigations with vector systems other than retroviruses. For instance, SV40 pseudovirions allow for highly efficient *MDR*1 gene transfer to hematopoietic cells (54,55). Alternatively, Epstein-Barr virus-based vectors can be designed that contain the *MDR*1 cDNA episomally in target cells (56). With such vector sys-

Table 1 Use of Drug-Resistance Genes to Confer Resistance on Bone Marrow

Gene	Selection	Reference
Multidrug resistance gene 1 (MDR1)	Multiple cytotoxic natural product drugs	Gottesman et al., 1995 (3)
Multidrug associated protein 1 (MRP1)	Multiple cytotoxic natural product drugs	Omori et al., 1999 (44)
Dihydrofolate reductase (DHFR)	Methotrexate and trimethotrexate	Flasshove et al., 1998 (1) Warlick et al., 2002 (70)
Cytidine deaminase	Cytosine arabinoside	Momparler et al., 1996 (74)
Glutathione transferase Yc	Melphalan, mechlorethamine, chlorambucil	Letourneau et al., 1996 (75)
Aldehyde dehydrogenase	Cyclophosphamide	Magni et al., 1996 (76) Moreb et al., 1996 (77)
O^6-methylguanine methyltransferase (O^6-MGMT)	Nitrosourea (BCNU)	Allay et al., 1995 (78)

tems life-long expression cannot be achieved because they fail to integrate into the genome. Conversely, for protection of hematopoietic cells during a series of chemotherapy cycles, sustained expression may not be required. Loss of *MDR*1 expression after the period of chemotherapy might in fact increase the safety of *MDR*1 gene therapy.

With the use of retroviral vectors, functional drug resistance of hematopoietic progenitor cells transduced by *MDR*1 vectors was first demonstrated in tissue culture (37) and recently in mice transplanted with transduced human progenitor cells (57). As a result, recipient animals can be treated safely with intensified chemotherapy following reinfusion of *MDR*1-transduced cells (58).

The efficiency of chemoprotection during antineoplastic chemotherapy is questionable if the cancers themselves express high amounts of P-glycoprotein. To further enhance the efficiency of this approach a vector has been designed that contains a mutant *MDR*1 cDNA (59). A point mutation in amino acid position 938 by which phenylalanine is replaced by alanine has little effect on the substrate specificity of P-glycoprotein but abolishes the reversing effect of a chemosensitizing agent, trans(E)-flupenthixol (60). Such vectors can be useful to overcome multidrug resistance in cancer cells, which are protected by wild-type P-glycoprotein, whereas hematopoietic cells are protected by the mutant gene even in the presence of anticancer drug and chemosensitizer.

Treatment of *MDR*1-transduced hematopoietic cells with anticancer drugs in tissue culture increases expression of *MDR*1 mRNA and P-glycoprotein in the surviving cell population as a result of clonal selection (61). Moreover, *MDR*1 transduced hematopoietic cells exposed to high concentrations of anthracyclines or colchicine prior to transplantation maintain their ability to engraft and rescue recipient mice from otherwise lethal irradiation (62). These animal studies provide useful models for the optimization of conditions for clinical applications.

Several of the drug resistance genes have been used to protect hematopoietic cells from drugs used in anticancer treatment. As has been seen with *MDR*1, chemoprotection of hematopoietic progenitor cells and a selective advantage in vitro were demonstrated following transduction by mutated DHFR cDNAs that confer resistance to methotrexate and trimetrexate (1,63–66). Williams et al. (67), Cline et al. (68), and Vinh et al. (69) demonstrated protection of recipient animals from lethal doses of methotrexate. Human $CD34^+$ cells can be transduced efficiently with a DHFR vector (1).

Mice transplanted with DHFR-transduced hematopoietic cells display resistance against high levels of trimetrexate, which can be transferred to a second generation of transplant recipients (70). Interestingly, mice can be transplanted with low amounts of DHFR-transduced cells following mild total body irradiation at a reduced dose of 1 Gy and acquire methotrexate resistance by treatment with this drug for 60 days following transplantation (71). Retransplantation experiments performed with dihydrofolate reductase (72) gave results comparable to those obtained with *MDR*1 (16); both genes facilitate increased levels of resistance after several cycles of transplantation and drug treatment of recipient animals.

In vivo selection of retrovirally transduced hematopoietic cells has convincingly been demonstrated with the DHFR as a selectable marker (73). In this study mice were transplanted with DHFR-transduced bone marrow cells. Drug treatment resulted in significantly increased expression in granulocytes, erythrocytes, platelets, and T- and B-lymphocytes. Secondary recipients revealed that selection had occurred at the stem cell level.

Resistance to another antimetabolite drug, cytosine arabinoside, which is a major component of treatment for acute leukemias, is conferred by cytidine deaminase. Hematopoietic cells were rendered resistant to cytosine arabinoside by transfer of this gene (74).

Different patterns of chemoresistance can be attributed to various drug resistance genes. For instance, the *MRP*1 gene is genetically and functionally related to *MDR*1. Retroviral transfer of *MRP*1 resulted in resistance to doxorubicin, etoposide, and vincristine (4). However, since binding and transport of inhibitors to *MDR*1 may be different from *MRP*, transfer of this gene may be useful if naturally occurring resistance due to *MDR*1 overexpression in cancer cells has to be overcome to allow for effective chemotherapy of an *MDR*1-expressing cancer.

Resistance to alkylating agents is multicausative, and several genes may be useful as selectable markers. Retroviral transfer of a rat glutathione S-transferase Yc cDNA to hematopoietic cells conveyed moderate resistance to melphalan, mechlorethamine, and chlorambucil (75). Resistance to cyclophosphamide or 4-hydroperoxycyclophosphamide, respectively, could be conferred on hematopoietic cells by transfer of aldehyde dehydrogenase with the use of retroviral vectors (76,77). Leukemic or primary hematopoietic cells were rendered resistant to BCNU by retroviral transfer of a human O^6-alkylguanine-DNA alkyltransferase cDNA (2,78,79). Transplantation of transduced bone marrow cells rescued recipient animals from the toxicity of nitrosoureas (80). In particular, nitrosourea-induced severe immunodeficiency can be overcome by transduction of immature progenitor cells (80,81). Furthermore, resistance to nitrosoureas in combination with an inhibitor of O^6-alkylguanine-DNA alkyltransferase, a key enzyme involved in naturally occurring resistance to nitrosoureas, could be conferred by retroviral transfer of a mutated O^6-methylguanine DNA methyltransferase cDNA (26). This approach protected mice from lethal drug doses and allowed selection of transduced hematopoietic progenitor cells.

To widen the range of anticancer drugs to be inactivated by gene therapy, vectors have been constructed for coexpression of two different drug resistance genes. For instance, a vector containing *MDR*1 and O^6-alkylguanine-DNA-alkyltransferase rendered human erythroleukemia cells resistant to the MDR1 substrates, colchicine and doxorubicin, as well as to alkylating agents, N-methyl-N-nitrosourea and temozolomide (82).

A different approach to utilize vectors that allow for coexpression of two genes is to include a second gene that may

enhance the efficacy of a selectable marker gene. This has been shown by construction of a vector that contained an MRP1cDNA and a cDNA encoding γ-glutamyl-cysteine synthetase, the rate-limiting enzyme of glutathione biosynthesis (83). Resistance to substrates of MRP1 was thereby increased due to elevated glutathione levels in transduced cells. The MRP1 transporter extrudes glutathione-conjugated compounds from the cell; hence, elevated concentrations of glutathione increase the transport of potential substrates.

Based on experiments in tissue culture and animal models, early clinical trials on transfer of the *MDR*1 gene to hematopoietic progenitor cells have been conducted (84–86). Bone marrow or peripheral blood progenitor cells from patients suffering from advanced neoplastic diseases were retrovirally transduced and reinfused after high-dose chemotherapy (87–89). These studies revealed that transduction efficiencies using *MDR*1 vectors as detected in bone marrow or peripheral blood of patients tended to be low, and varied from one patient to another. Notably, in two recent studies enrichment of *MDR*1-transduced cells was observed following treatment with etoposide or paclitaxel, respectively (90,91). The studies confirm the concept that the human multidrug resistance gene can serve as a drug-selectable marker gene in vivo in the hematopoietic system. However, gene transfer procedures and selection strategies need to be improved to efficiently protect human hematopoietic cells from the cytotoxicity of drug treatment. In particular, clinical studies should be conducted with novel vector constructs and improved culture conditions that allow for increased transduction rates.

Improvements in vector design have been suggested by several groups. Using the multidrug resistance gene, Metz et al. (92) showed that retroviral vectors derived from Harvey viruses can be substantially shortened without reduction of gene transfer efficiency, thereby increasing the maximum size of the packaged gene of interest. By systematic analysis of the U3-region of various 5′-long-terminal repeats, Baum et al. (93) optimized *MDR*1 transfer to hematopoietic cells. Notably, transfer to immature hematopoietic progenitor cells, which are generally difficult to transduce, was improved (94). More recent improvements of posttranscriptional processing led to a vector that reliably ensured *MDR*1 expression and drug efflux in human hematopoietic cells following an in vivo passage in immunodeficient mice (95). Other vector systems used for chemoresistance gene transfer to hematopoietic cells include adeno-associated virus vectors (96) or liposomes (97) (see Sections IV and V.).

New vector constructs increase the efficiency of gene transfer to hematopoietic cells but do not necessarily ensure gene expression for sustained periods. A major obstacle to long-term gene expression is the limited lifespan of some transduced cell clones. Since only hematopoietic stem cells have the capability of self-renewal, the lifespan of progeny generated by more differentiated progenitor cells is limited. Berger et al. (98) have shown that expansion of cells with cytokines, particularly with interleukin-3 (IL-3), reduces the frequency of long-term culture-initiating cells (LTC-IC), which correlated with reduction of Rhodamine-123 efflux from immature progenitor cells. In accord with these findings, Schiedlmeier et al. (99) reported that IL-3-stimulated hematopoietic cells engrafted more poorly than cells grown in the presence of other growth factor combinations. Both studies resulted in efficient retroviral *MDR*1 transfer to primitive human progenitor/stem cells.

A different approach to improve the utility of selectable markers is to coexpress two drug resistance genes, thereby conferring resistance to a broad range of cytotoxic agents. To this end, mutated dihydrofolate reductase has been coexpressed with *MDR*1 or with thymidylate synthase (100,101), and *MDR*1 has also been expressed with O^6-methylguanine-DNA-methyltransferase (102,103). Coexpression of glutathione S-transferase and cytidine deaminase rendered cells resistant to cytosine arabinoside and alkylating agents such as melphalan and chlorambucil (104). These compounds are used in chemotherapy of malignant lymphomas.

Alternatively, a dominant-positive selectable marker gene can be coexpressed with a negative selectable marker such as thymidine kinase from Herpes simplex virus (HSV-TK) (105,106). The latter approach allows selective elimination of transduced cells. Such an approach may increase the safety of gene transfer if cancer cells contaminating hematopoietic cell preparations are inadvertently rendered drug-resistant, or if transduced cells become malignant (51,107). Selective killing of *MDR*1-HSV-TK transduced cells in vivo has been demonstrated (106). Thymidine kinase may not only facilitate selective killing of cancer cells but instead increase the efficacy of certain selectable marker genes. A bicistronic vector in which thymidine kinase was combined with dihydrofolate reductase displayed enhanced resistance as compared to a construct that contained a neomycin phosphotransferase instead of thymidine kinase (108). The authors concluded that thymidine kinase may be useful to salvage thymidine.

To increase the safety of gene therapy of cancer, drug resistance genes may be combined with cDNAs that specifically eliminate cancer cells. This has been demonstrated for chronic myeloid leukemia (CML), which is characterized by a specific molecular marker, the BCR/ABL gene fusion. A vector has been constructed that combined a methotrexate-resistant dihydrofolate reductase with an antiBCR/ABL antisense sequence (109). Transfer of this vector to CML cells led to the restoration of normal cellular function of BCR/ABL cDNA+ cells due to reduced levels of transcripts while conferring drug resistance.

In addition to improvement of gene therapy of cancer, drug resistance genes may be helpful for gene therapy of nonmalignant diseases if increased gene expression is desired. In fact, there is considerable interest in using drug selectable marker genes to introduce and enrich otherwise nonselectable genes in target organs. Gene therapy, although thought to bear the potential of curing genetically determined diseases, is frequently hampered by low gene expression in target organs. This is particularly true for hematopoietic disorders because the efficiency of gene transfer is often limited, and stable expression of transgenes in bone marrow has been found difficult to accomplish.

For instance, Gaucher disease is characterized by accumulation of a glucosylceramide in glucocerebrosidase-deficient hematopoietic cells. These patients suffer from skeletal lesions, severe hepatosplenomegaly, anemia, and disorders of the central nervous system. While it is possible to efficiently transduce a glucocerebrosidase cDNA to hematopoietic progenitor cells (110,111), expression levels tend to decrease after several weeks or months in vivo because of silencing or limited lifespan of the transduced cells' progeny. To increase expression of glucocerebrosidase in vivo, Aran et al. (112) constructed a transcriptional fusion between *MDR*1 and the glucocerebrosidase gene. Increased expression of the latter gene was achieved by selection with cytotoxic substrates of P-glycoprotein. Appropriate selection strategies allowed complete restoration of the underlying genetic defect in cells from Gaucher patients (113). Transduction of such bicistronic vectors into hematopoietic stem cells might allow treatment of patients by chemotherapeutical elimination of non-transduced cells that continue to synthesize or store glucosylceramide. Moreover, following chemotherapy, the numbers of genetically corrected hematopoietic progenitor cells should increase in bone marrow to maintain physiological numbers of mature granulocytes, monocytes, and lymphocytes in peripheral blood. Recently, in vivo selection for cells expressing glucocerebrosidase was demonstrated with a vector containing the selectable marker gene, DHFR (114).

Similarly, bicistronic vectors that facilitate coexpression of *MDR*1 and α–galactosidase A have been engineered (115). Defects of α–galactosidase A are the cause of Fabry disease, a globotriaosylceramide storage disorder that affects the skin, kidneys, heart, and nervous system. Other applications for bicistronic fusions include immunological disorders such as chronic granulomatous disease and X-linked or adenosine deaminase (ADA) deficiency-related severe combined immunodeficiency (SCID) syndromes. For treatment of these diseases, vectors have been constructed that contain a gp91phox or an ADA cDNA (116–119). DHFR was used as a selectable marker gene in a bicistronic vector for correction of α_1-antitrypsin deficiency (120). Further discussion of the use of bicistronic vectors is found in Section III.

A different strategy to exploit the *MDR*1 gene as a drug-selectable marker for correction of ADA deficiency was described by Germann et al. (121). In this study, both genes were fused to a single cDNA encoding a bifunctional chimeric protein. This approach, however, cannot be used if the two proteins are physiologically located in different cellular compartments.

Another system in which selectable markers may be useful is in the skin. It is possible to grow keratinocytes in culture and introduce the *MDR*1 gene via retroviral vectors. Such keratinocytes are resistant to MDR drugs in vitro, and when transplanted on keratinocyte ''rafts'' to recipient animals, they remain resistant to colchicine, which can be applied as an ointment. If colchicine is withdrawn, transplanted keratinocytes are gradually replaced by nontransduced host skin; in the presence of selection, the transplanted keratinocyte graft is maintained. It should be possible in such a system to introduce other nonselectable genes via bicistronic vectors to serve as a source of protein to treat a genetic defect in the skin or elsewhere in the host (122).

While MDR vectors are well characterized in vitro, their usefulness in vivo has still to be established. We have recently demonstrated in a large-animal model that expression of a nonselectable gene that had been undetectable for more than one year can be recovered by coexpression with a drug-selectable marker, *MDR*1 (17). In this study, high levels of the nonselectable, therapeutic gene were achieved in peripheral blood and bone marrow following treatment of the animal with paclitaxel. The toxicity of this treatment, however, was considerable. Thus, further investigations in animal models are needed to optimize selection strategies in live animals.

Detection of the function of transferred genes may be difficult if normal animals are utilized because of the activity of the respective endogenous gene product. To circumvent this difficulty, ''knock-out'' animals whose gene has been inactivated by targeted disruption can serve as useful models. For instance, mice whose α–galactosidase gene has been disrupted may be helpful to characterize a bicistronic vector in which *MDR*1 is combined with the respective human gene for correction of Fabry disease (123). Another alternative is to use marking genes that are not physiologically expressed at high levels in normal tissues. To characterize bicistronic vectors containing *MDR*1, this gene has been coexpressed with a green fluorescent protein or β–galactosidase (124).

These model systems should help to improve protocols for efficient drug selection and to identify strategies for selection at limited systemic toxicity. For instance, addition of P-glycoprotein inhibitors at low concentration to cytotoxic drugs may increase the stringency of drug selection, thereby allowing use of anticancer drugs at low concentrations for selection (113).

III. BICISTRONIC VECTORS CONTAINING SELECTABLE MARKERS

Although coexpression of two proteins can be achieved through the use of separate promoters, the coexpression is frequently uncoupled due to promoter interference or shutoff of gene expression from one of the promoters, which causes the selected cells not to express the desired protein. To overcome this problem, the selectable marker may be expressed with the therapeutic gene as a translational or transcriptional fusion. A therapeutic protein can be directly linked to the carboxylterminus of the multidrug transporter P-glycoprotein (P-gp). The resulting fusion protein possesses functions of both P-gp and the target protein (125). Since P-gp is an integral membrane protein that functions on the cell plasma membrane, unless two proteins can be separated by a posttranslational proteolytic modification, the expressed target protein will be associated with the plasma membrane regardless of its normal cellular location. Thus, even though translational fusions guarantee protein coexpression, their potential is limited. On the other hand, transcriptional fusions, e.g., using

bicistronic or polycistronic mRNA to encode more than one cDNA, may prove to be more generally applicable.

A. *MDR*1 Bicistronic Vectors Containing Internal Ribosome Entry Sites (IRES)

A DNA segment corresponding to one polypeptide chain plus the translational start and stop signals for protein synthesis can be loosely defined as a cistron. An mRNA encoding only a single polypeptide is called monocistronic mRNA; if it encodes two or more polypeptide chains, it may be called bicistronic or polycistronic mRNA. Almost all eukaryotic mRNA molecules are monocistronic. Initiation of translation of eukaryotic mRNA is mediated by cap-binding protein that recognizes a methylated guanosine cap at the 5′ terminus of mRNA. However, some viral mRNA molecules transcribed in eukaryotic cells are polycistronic. They can use a cap-independent mechanism to initiate translation in the middle of mRNA molecules. For picornavirus, this cap-independent internal initiation of translation is mediated through a unique internal ribosome entry site (IRES) within the mRNA molecule (126,127).

Identification of IRES sequences has led to the development of bicistronic vectors that allow coexpression of two different polypeptides from a single mRNA molecule in eukaryotic cells (128,129). Using a bicistronic vector containing an IRES to coexpress a target gene and a selectable marker has several advantages. First, since two polypeptides are translated from the same mRNA molecule, the bicistronic vector guarantees coexpression of a selectable marker and a second protein. Secondly, bicistronic mRNA allows two polypeptides to be translated separately. Thus, this system does not compromise the correct intracellular trafficking of proteins directed to different subcellular compartments. In addition, using a bicistronic vector, expression of a target gene is proportionate to the expression of a selectable marker. Hence, expression of a target protein can be achieved quantitatively by applying selections of different stringencies.

To demonstrate coexpression of a dominant selectable marker with a therapeutic gene using a bicistronic vector, our laboratory has coexpressed P-gp with glucocerebrosidase (112,113), β-galactosidase (115), adenosine deaminase (118), a subunit of the NAPH oxidase complex (116,117), the shared gamma chain of the interleukin receptors (119), and a hammerhead ribozyme targeted to the U5 region of HIV-1 LTR (130). In those experiments, *MDR*1 served as a selectable marker linked to the target gene by an IRES from encephalomyocarditis virus (EMCV) and constructed in a retroviral vector containing Harvey sarcoma virus LTR (131). Two configurations, in which *MDR*1 is placed either before or after the IRES, have been examined in some cases. As demonstrated in those experiments, P-gp and the target gene are coexpressed in the cells selected using cytotoxic P-gp substrates, such as colchicine or vincristine; the expressed target proteins are functional as detected using in vitro, or ex vivo analysis. In one case, using subcellular fractionation, we have demonstrated that P-gp and glucocerebrosidase are translocated separately to the cell plasma membrane and lysosomes, indicating correct intracellular protein trafficking (112). The demonstration that a noncoding RNA, such as a hammerhead ribozyme, can function even though tethered to an mRNA encoding a functional *MDR*1 provides an additional powerful way to use bicistronic vectors (130).

Another approach to the use of *MDR*1-based bicistronic vectors is to develop ''suicide'' vectors for cancer gene therapy. Using *MDR*1 to protect bone marrow cells from cytotoxic drugs represents a promising approach to improve cancer chemotherapy. However, contaminating cancer cells may be inadvertently transduced with *MDR*1, or transduced bone marrow cells may accidentally develop new tumors. In those cases, overexpression of P-gp could cause multidrug resistance in inadvertently transduced tumor cells that contaminate bone marrow, or in any transduced cells that later become malignant. A bicistronic ''suicide'' vector developed in this laboratory links P-gp expression with herpes simplex virus thymidine kinase (TK) expression (105,106). Thus the cells containing this vector can be eliminated through ganciclovir treatment.

A third approach is to link two drug resistance genes together using a bicistronic vector system to extend the ability of the vector to confer drug resistance. Examples include the use of *MDR*1 with dihydrofolate reductase that confers methotrexate resistance (132), *MDR*1 plus methylguanine methyltransferase (MGMT) that confers resistance to certain alkylating agents (102,103,133), and *MRP*1 plus gamma-glutamylcysteine synthetase that confers resistance to alkylating agents as well (83).

Finally, bicistronic vectors can be used to introduce marker genes into selected cells. For example, *MDR*1 vectors containing green fluorescent protein or β-galactosidase have been constructed to determine the efficiency of expression of the target gene in transduced and *MDR*1 selected cells (124).

B. Efficiency of IRES-dependent Translation

Using an IRES to generate a bicistronic mRNA ensures coexpression of two different proteins. However, IRES-dependent mRNA translation (or cap-independent translation) is less efficient than cap-dependent translation, so that the two proteins are not expressed in equal amounts. It has been shown that in a monocistronic vector, insertion of an IRES upstream from an open reading frame of either P-gp or dihydrofolate reductase (DHFR) reduces the translation efficiency by 2- to 10-fold (129,134). Using a bicistronic vector, expression of *neo* in the position downstream from the IRES is 25% to 50% of that observed when *neo* is in the upstream position (128). The asymmetric expression pattern of the bicistronic vector results in a significant difference in *MDR*1 transducing titer between a configuration with P-gp placed before the IRES and a configuration in which P-gp is placed after the IRES. We have found that the apparent titer of a bicistronic vector containing *ADA-IRES-MDR*1 was only 7% of the titer of a bicistronic vector containing *MDR*1*-IRES-ADA* (118). Similar reductions in *MDR*1 transducing titer and in expression of

the nonselected downstream gene was seen with *MDR*1-β-galactosidase bicistronic vectors too (115). The apparent *MDR*1 transducing titer of the retrovirus is based on the drug resistance conferred by expression of P-gp as the result of retroviral infection; thus the viral titer is proportional to the P-gp expression level. Insufficient expression of P-gp is unable to protect the cells from cytotoxic drug selection. To achieve P-gp expression at the same level, the lower efficiency of translation would have to be compensated for by a higher level of transcription, which can occur only in a minority of the cells in the transduced population. This may account for the apparent lower *MDR*1-transducing titer of bicistronic vectors with a configuration of P-gp placed after the IRES. On the other hand, when cells express P-gp at the same level (i.e., the cells survived vincristine or colchicine selection at the same concentration), ADA expressed from *ADA-IRES-MDR*1 is 15-fold higher than the ADA expressed from *MDR*1-*IRES-ADA*. This difference is probably due to a combination of the lower translation efficiency of ADA located downstream from the IRES and the high transcription level of *ADA-IRES-MDR*1 as the result of vincristine selection. A similar asymmetric expression of P-gp and human β−galactosidase A is also observed in NIH3T3 cells, where the difference is about 8-fold.

IRES-dependent translation is a complex process, in which mRNA containing IRES interacts with various cellular proteins, including IRES transacting factors [reviewed in Hellen and Sarnow (135)]. The efficiency of IRES-dependent translation can be affected by the cell type (136), IRES origin (137,138), and the size and structure of a particular mRNA molecule. We have found that the titer of retrovirus containing pHa-*MDR*1 was higher than pHa-*MDR*1-*IRES-ADA*, even though P-gp translation was cap-dependent in both cases. P-gp expressed from pHa-*MDR*1 was also at a higher level in a vincristine resistant cell population than the P-gp expressed from pHa-*MDR*1-*IRES-ADA*. A possible explanation for the relatively low retroviral titers observed is RNA instability or alternative splicing, since no DNA rearrangement was detected by Southern blot analysis of the transduced cells using an *MDR*1 probe.

In addition to IRESes derived from viruses, several IRES elements have been identified in human genes. Those IRESes play important roles in cell cycle-dependent or stress-response translation regulation [reviewed in Sachs (139)]. In contrast to viral IRESes, human IRESes are shorter and are complementary to 18s rRNA [reviewed in Mauro and Edelman (140)]. It has been found that a 9-nt sequence from the 5′-UTR of the mRNA encoding the Gtx homeodomain protein can function as an IRES. Ten linked copies of the 9-nt sequence are 3- to 63-fold more active than the classical EMCV IRES in all 11 cell lines tested (141). Similarly, an IRES isolated from the human EIF 4G gene also exhibits 100-fold more IRES activity than EMCV IRES in 4 different cell lines (142). In addition to higher efficiency and smaller size, translation from a human IRES can be regulated by cellular events (142), which may be advantageous for certain cancer gene therapies.

C. Flexibility Using Bicistronic Vectors in Coordinating Expression of Selectable Markers and a Therapeutic Gene

Selectable bicistronic vectors provide great flexibility in coordinating expression of a selectable marker, such as P-gp, and a therapeutic gene. The low translation efficiency of the IRES results in asymmetric expression of genes positioned before and after the IRES. This asymmetric expression pattern makes it possible to alter the relative expression level of a therapeutic gene and P-gp to achieve maximum therapeutic effects while applying minimal selective pressure using a cytotoxic drug. By choosing different configurations, i.e., placing *MDR*1 before or after the IRES, we can select cells expressing a therapeutic gene at either a low level (*MDR*1 before the IRES) or a high level (*MDR*1 after IRES).

In addition, expression of a therapeutic gene can also be achieved at a desired level by altering the selection conditions. The degree of multidrug resistance conferred by P-gp corresponds to the amount of P-gp expressed on the plasma membrane. Using a bicistronic vector, the expression of a target gene is proportional to the expression of P-gp, which is directly linked to the selection conditions. In a highly stringent selection, instead of increasing the concentration of cytotoxic drug, P-gp reversing agents can also be applied in combination with low concentrations of cytotoxic drugs (143). P-gp reversing agents, also known as chemosensitizers, are noncytotoxic hydrophobic compounds that interact with P-gp and cause a direct inhibition of P-gp function. In the presence of a P-gp reversing agent, most P-gp-expressing cells are killed by the cytotoxic drug unless they express a large amount of P-gp to overcome the inhibitory effects. Using a combination of cytotoxic drug and chemosensitizer allows selection of cells expressing the therapeutic gene at a high level without need for a high concentration of cytotoxic drug. This strategy is especially desirable for an in vivo selection in which avoiding systemic toxicity is essential.

High expression of the target gene can be selected using cytotoxic drugs, cytotoxic drugs combined with chemosensitizers, or the vector configured to place the target gene placed before the IRES. However, those approaches also reduce the overall number of cells that can survive the selection. Nevertheless, using a minimum concentration of drug, the selectable bicistronic vector provides options for selecting a large population of cells with low expression of the target gene, or a small population of cells with high expression of the target gene. Both options may be useful for gene therapy. For instance, ADA levels in normal individuals occur over a very broad range. Heterozygous carriers can be immunologically normal even with as little as 10% of the normal amount of ADA [reviewed by Blaese (144)]. Expression of ADA at a low level in a large number of cells may prove sufficient to treat SCID. On the other hand, high ADA-expressing lymphoid cells, even through present as a small percentage of total cells, are also able to correct the SCID syndrome due to a beneficial by-stander effect (145). In gene therapy applications, the choice of the approach depends on the thera-

peutic strategy for a specific disease. Experiments on animal models are essential to prove the concepts that underlie gene therapy using selectable markers such as *MDR*1.

IV. NONRETROVIRAL AND EPISOMAL VECTORS EXPRESSING SELECTABLE MARKERS: AAV, SV40, AND EBV

Efficient delivery of a therapeutic gene to the appropriate target cells and its subsequent maintenance and expression are important steps for successful gene therapy. Genes introduced into cells are rapidly lost unless there is a mechanism to retain these genes within the nucleus and to ensure that the genes are also replicated and partitioned into daughter cells during cell division. Long-term expression of the transgene within cells can be achieved either via the integration of the transferred DNA into the host genome or maintenance of the introduced DNA as an autonomously replicating extrachromosomal element or episome. In either case, inclusion of a drug-selectable marker, like the *MDR*1 gene, in the construct would ensure that rapidly dividing cells containing the transgene are given a selective growth advantage.

Delivery modalities can be viral or nonviral. Retroviral gene transfer, one of the most exploited systems for gene transfer into actively dividing cells, has been discussed earlier in this chapter while liposomal gene delivery will be discussed later in the chapter. In this section, nonretroviral and/or episomal vectors expressing selectable markers will be described.

A. AAV

In addition to retroviruses, adeno-associated virus (AAV) can also facilitate integration of the transgene into the host genome. Unlike retroviruses, AAV was found to integrate preferentially into a specific site on chromosome 19 (146). AAV is a naturally defective, nonpathogenic, single-strand human DNA parvovirus. For productive infection and viral replication, coinfection with helper viruses, e.g., adenovirus, herpesvirus, or vaccinia virus are required. In the absence of a helper virus, AAV establishes latency in the host by integrating itself into the host genome. AAV has a broad host range and is also able to infect both dividing and nondividing cells (147). Hence recombinant AAV (rAAV) vectors have been exploited as alternative vehicles for gene therapy.

AAV-based vectors (148) are simple to construct, requiring only that the viral inverted terminal repeat (ITR) (which are 145 nucleotides each) is upstream from the gene of interest. Other important viral genes like rep (involved in replication and integration) and cap (encoding structrual genes) can then be supplied in trans. One disadvantage with such rAAV vectors is that site-specific integration of the gene of interest into the host genome is not observed (96). This is probably because the rep gene, which is important for mediating site specific integration in the absence of helper viruses, is not included in the construct with the gene of interest. Nonetheless, rAAV has been successfully applied to the delivery of various genes into a variety of tissues and persistence of transgene expression in these nondividing tissues, was reported (149–154). Baudard et al. (96) demonstrated that in rapidly dividing cells, continuous selective pressure is necessary to sustain gene expression in cells. *MDR*1 was used as the selectable marker in this study. Being among the smallest DNA animal viruses (~20 nm in diameter), another disadvantage of the AAV system is its limited packaging capacity since it can accomodate only aproximately 4.7 kb of the gene of interest. As such, a small and efficient promoter would be required to drive the expression of large genes. One such promoter is the AAV p5 promoter, which, together with the ITR, forms a 263-base pair cassette capable of mediating efficient expression in a CF bronchial epithelial cell line (149,150). Baudard et al. further demonstrated that the reduction of the p5 promoter-ITR cassette to 234 bp was also able to promote efficient gene expression (96).

B. SV40

Vectors that facilitate extrachomosomal replication have some advantages. High gene expression is often observed in such vectors. This could be a result of vector amplification, promotion of nuclear localization and retention, as well as transcriptional activation by viral genes involved in episomal replication. Selective pressure using selectable markers like the *MDR*1 gene, however, is necessary to maintain these episomes in actively dividing cells. Thus, another potential advantage of using episomally replicating vectors is that since they are not integrated into the cells, one could potentially extinguish expression at will by withdrawing selective pressure to replicating cells. Episomally replicating vectors can be easily created by the inclusion into the vector design of replicons that can be derived from DNA viruses like the Simian Virus 40 (SV40) (155), Epstein-Barr virus (EBV) (156) and the BK virus (157–159). Such replicons usually comprise a viral origin of replication as well as a viral gene product that is important for maintaining extrachromosomal replication.

SV40 is a 5.2 kb DNA papovavirus that was discovered as a harmless contaminant in early preparations of the Salk polio vaccine (160,161). SV40 is a double-stranded circular 5.2 kb DNA simian virus. It is a nonenveloped virus that belongs to the papovavirus family. The SV40 capsid is composed of 72 pentamers of the major capsid protein VP1, which are tied together through their carboxy-termini. VP2 and VP3, which share 234 amino acids at their carboxy-termini, connect the minichromosome core to the axial cavities of VP1. It has been suggested that correct interpentamer bonding is facilitated by host chaperones (162,163). SV40 infection begins with the virus binding to its primary receptor, the major histocompatibility complex class I (MHC class I), without internalization of the receptors. The entry is mediated by caveolae and the virus is transported to the Golgi membranes. Its pathway extends to the endoplasmic reticulum (ER), where it is disassembled. The mechanism by which the virus reaches the nucleus is not yet known (164–167). The wild-type virus is unable to replicate its DNA in rodent cells; therefore no progeny

virions can be produced in these cells (168). Infection of SV40 wild-type virus in cells can result in the integration of viral DNA into the host chromosome, permitting transmission of expression to daughter cells (169). Some reports associate SV40 DNA (specifically the T-antigen) with human tumors, mainly based on the presence of sequences from SV40 wild-type in some brain tumors and melanomas (170,171).

The two major SV40 delivery systems are vectors that use SV40 sequences or the wild-type virus as a helper, and vectors that are packaged in vitro, with no SV40 sequences and in which the wild-type virus is not present. SV40 has numerous advantages as a gene-delivery vehicle (54,172,173): it is able to infect a wide variety of mammalian cells, including human cells, and to express its genes in these cells; the vector system has an ability to deliver untranslated RNA products; the gene expression may be transient or stable in cell lines, depending on the specific SV40 system that is used; and episomal replication in SV40 virus requires the SV40 replication origin as well as the large T antigen (T-Ag), which activates the replication origin . Such episomal replication can generate more than 105 copies per cell of recombinant plasmids (174). Using the SV40 delivery systems, no immune response is expected, as well as no inflammatory reaction.

Replacing the late or early region with a foreign gene can generate SV40 recombinant viral particles (169,172,175–181). These are then propagated using either wild-type, or a temperature-sensitive mutant of SV40 as helper, or via a viral producer cell line, COS7, that stably expresses an origin-defective SV40 mutant and is capable of supporting the lytic cycle of SV40. Multiple infections result in higher titers of the virus — up to 10^{10} infectious units/ml. It has been demonstrated that when the large T-antigen (T-Ag) gene is replaced with a reporter gene, replication-deficient recombinant SV40 viruses can be produced and can mediate gene transfer *in vivo*. Reporter gene expression was detectable for about 3 months without selection. Present SV40 vectors of the first type have most of the viral coding sequences removed, retaining only the packaging sequences, the polyadenylation signal, and the early promoter of the virus, thus increasing the capacity for DNA to ~5.3 kb. The DNA from these vectors integrates into the genome of the target cells.

Rund et al. (54) demonstrated very efficient delivery (> 95%) of the drug-selectable marker, *MDR*1, into various murine and human cell types including primary human bone marrow cells (54). SV40 vectors efficiently deliver HIV-1-inhibitory RNAs using pol II or III promoters. Other vectors, which encode a variable fragment antibody recognizing HIV-1 integrase, inhibited HIV-1 infection in SCID mice. This system may prove to be useful in antiHIV-1 therapeutics. Fang et al. (182) reported a different packaging system for SV40 vectors where the vector carrying the gene of interest contains only the SV40 origin of replication (182). Instead of using wild-type SV40 viruses as helpers to package the recombinant vector, recombinant adenoviruses expressing SV40 capsids were used in COS7 cells. The helper adenovirus can be effectively heat-inactivated without adverse effect on the infectivity of the recombinant SV40 viruses due to the differential heat sensitivity of these two viruses. Strayer et al. (169) found evidence for integration of the recombinant gene or parts of it, a few days after transduction in random sites, which might explain the long-term expression of this system.

Pseudovirions can transfer the gene of interest to a variety of cells (including hematopoietic cells) with high efficiency, but their clinical applicability is currently limited by the presence of wild-type SV40 sequences. The in vitro, method of preparing helper-free SV40 vectors utilizes the SV40 viral late proteins, VP1, VP2, VP3 and agno or VP1 only (183–186). Nuclear extracts of baculovirus-transduced *Spodopterafrugiperda* (Sf9) insect cells that include these proteins are incubated with supercoiled plasmid DNA in the presence of 8 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM ATP to form the SV40 in vitro, packaged vectors (55). Such in vitro, assembly allows larger DNA plasmids (up to 17.6 kb) to be packaged very efficiently, with no need for SV40 sequences. We have demonstrated very efficient delivery of the *MDR*1 (*ABC B1*), *MXR* (*ABC G2*), and *MRP*1 (*ABC C1*) genes, which can confer multidrug resistance on virtually all cell types (human, murine, and monkey cell lines), in addition to delivery of the *GFP* gene as a reporter (187). The expression of both *MDR*1 and *GFP* genes is dose dependent. The alteration in the level of expression suggests that MHC class I receptors play an important role in determining the efficiency of transduction. *MDR*1 constructs that carried a promoter with an intron demonstrated higher expression than those without the intron. In low-expressing MHC class I cell lines, the CMV promoter produced more P-gp expression compared with the SV40 promoter. In vitro packaged-*GFP* vectors that carried the CMV promoter consistently confirmed higher expression than those that carried the SV40 promoter. Expression was transient up to 21 days, but did last for 3 months under colchicine selection for MDR1 and was lost soon after selection was withdrawn (55).

The short-term expression of the SV40/*MDR*1 in vitro, vectors may be an advantage for use in chemoprotection. Long-term expression beyond the chemotherapy period is undesirable, and may put patients at risk for treatment-induced myelodysplasia or secondary leukemia. The SV40/*MDR*1 vectors that are prepared in vitro, may provide not only a safe vehicle for gene delivery but will also potentially avoid the problem of persistent bone marrow drug resistance in cancer patients.

C. EBV and Other Episomal Vectors

Episomal vectors based on EBV are also being developed for gene therapy purposes. EBV is a human B-lymphotropic herpesvirus that resides asymptomatically in more than 90% of the adult human population by establishing latency and maintaining its genome episomally (188). The life cycle of EBV comprises two phases, a lytic and a latent phase. During the lytic phase, EBV DNA replicates via a rolling circle intermediate to achieve a 1000-fold increase in copy number. The origin of replication, Ori Lyt, and the transacting element ZEBRA are required for the lytic replication. Rolling circle replication results in the formation of linear head-to-tail con-

catamers. The presence of the EBV terminal repeat (TR) sequence causes cleavage of the concatemerized DNA to molecules of about 150–200 kbp, which are then packaged into virions. Upon infection into a permissive cell, the viral DNA circularizes by ligation of TR. Latency is established in the cells by episomal replication of the circular DNA.

Episomal replication in EBV is maintained by two elements interacting to ensure that the viral genome is retained within the nucleus, efficiently replicated and partitioned into daughter cells. Although the copy numbers of episomal viral DNA varies from 1–800, only between 4–10 episomal copies per cell are usually observed using vectors containing EBV OriP and EBNA-1 (189). Unlike other episomal vector systems, very low rates of spontaneous mutation have been observed with EBV-based episomal vectors (190). The *cis*-acting element responsible for episomal replication is a 1.8 kb OriP while the transacting element is EBNA-1. OriP comprises two distinct sequence motifs, the dyad symmetry motif (DS) from which replication is initiated and the family of repeats (FR) that serves as a replication fork barrier. Interaction of EBNA-1 with DS initiates bidirectional replication, while binding of EBNA-1 to FR enhances transcription from the episome and terminates DNA replication. EBNA is reported not to be oncogenic nor immunogenic. It evades the host immune system via the presence of the repeat motif, Gly-Ala, which was found to interfere with antigen processing and MHC class I-restricted presentation (191). These EBV episomal vectors replicate once per cell cycle (192) and are capable of stably maintaining human genomic inserts of sizes between 60–330 kb for at least 60 generations (193).

Banerjee and colleagues (194) developed a helper-dependent infectious recombinant EBV to evaluate the feasibility of using such a vector system to correct hereditary syndromes in B-lymphocytes already harboring the EBV virus latently. The EBV-containing target B-lymphocytes will supply EBNA-1 *in trans* for the episomal maintenance of the transgene. Hence only minimal cis-EBV elements for episomal replication (OriP), viral amplification (Ori Lyt), and packaging (TR) are included in their construct. The hygromycin resistance gene was included as a selectable marker in their vector. Infectious virions are generated by the producer cell line HH514. They demonstrated successful transfer of such infectious virions carrying the therapeutic gene, Fanconi anemia group C (FA-C) cDNA, into HSC536, a FA-C patient cell line. Upon selection with hygromycin, long-term (at least 6 months) correction of the Fanconi phenotype in vitro, was observed, as determined by cellular resistance to the cross-linking agent, diepoxybutane. They also observed that in the absence of selective pressure, their episomal vector is retained in rapidly dividing cells at a rate of 98% per cell division translating to a half-life of 30 days in cells doubling every 20 hours.

Our laboratory has been exploring the use of EBV episomal vectors containing only the OriP and EBNA-1 and carrying the selectable marker *MDR*1 as potential gene therapy vectors. Using the liposome formulation, DOGS/DOPE (1:1) (195), we successfully delivered the vector to various cultured cells as well as human $CD34^+$ stem cells. *MDR*1 was found to be expressed at a higher level in the episomal vector compared to its nonepisomal counterpart and more drug colonies were obtained upon selection. Episomal plasmids could be recovered in drug selected cells for many weeks (56).

Other episomally replicating vectors can be derived from BPV viruses (196) or the BK virus (158). Unfortunately, BPV vectors cannot be reliably maintained as episomes as they exhibit high spontaneous mutation rate (~1%), frequently undergoing integration, deletion, recombination, and rearrangements (197). Furthermore, BPV has a limited host range and BPV vectors cannot be efficiently maintained in human cells. Not too much is known about BK-virus-derived episomal vectors. Nonetheless, successful stable maintenance of episomal gene expression was reported in human transitional carcinoma cells using BK-based vectors but not EBV-based vectors, probably due to the differential tropism of BK and EBV viruses for human uroepithelial cells (157).

Various chimeric viruses have been developed to improve the efficiency of gene transfer as well as the maintenance of gene expression within target cells. These chimeric virus systems attempt to combine the favorable attributes of each vector system and overcome the limitations associated with each system. The episomal replication ability of EBV was exploited to produce both rapid and long-term high-titer recombinant retroviruses (up to 10^7 TU/ml) for efficient gene transfer into human hematopoietic progenitor cells (198,199). A novel adenoviral/retroviral chimeric vector was also reported in which an adenoviral delivery system was utilized to efficiently deliver both the retroviral vector and its packaging components, thereby inducing the target cells to function as transient retroviral producers capable of infecting neighboring cells. This system capitalizes on the superior efficiency of adenoviruses to deliver genes in vivo and the integrative ability of retroviruses to achieve stable gene expression (200). An EBV/HSV-1 amplicon vector system was also described that combines the efficiency of HSV-1 virus to transfer DNA into various mammalian cells, including the postmitotic neuronal cells and the ability of EBV to maintain genes episomally. This vector system contains the HSV-1 origin of DNA replication (oriS) and a packaging signal, which allow replication and packaging of the amplicon into HSV-1 virions in the presence of HSV-1 helper functions as well as EBV oriP and EBNA-1 (201). Another report describes the use of a similar HSV-1 amplicon system for efficient gene transfer, but AAV was included in their vector to achieve stable expression. This HSV/AAV hybrid vector contains OriS and packaging sequences from HSV-1, a transgene cassette that is flanked by AAV ITRs as well as an AAV rep gene residing outside the transgene cassette to mediate amplification and genomic integration of ITR-flanked sequences (202). An HVJ-liposome vector system reported by Dzau et al. (203) was utilized to improve the efficiency of liposome-mediated transfer of an EBV-episomally maintained transgene (204,205). This system exploits the fusigenic properties of the hemagglutinating virus of Japan (HVJ or Sendai virus) since envelope proteins of inactivated HVJ were found to mediate liposome-cell mem-

brane fusion and facilitate cellular uptake of packaged plasmid DNA, bypassing endocytosis and lysosomal degradation.

One of the limitations with using viral episomal systems is the limited host range of such vectors. Although EBV episomal vectors replicate well in various human and primate cells, they are unable to replicate in rodent cells, limiting their utility in gene therapy since testing of these vectors in rodent models is not easy. Nonetheless, it was found that large fragments of human genomic DNA (between 10–15 kb) can mediate autonomous replication if there is also a mechanism to retain them in the nucleus (206). Such vectors based on a human origin of replication were also found to be capable of replicating in rodent cells (207), probably due to the common host factors that drive their replication. A hybrid class of vectors was thus developed, which employs a human origin of replication to mediate vector replication as well as the EBV FR and EBNA-1 gene product to provide nuclear retention functions. [see Calos (208)]. EBNA-1 binding to the FR of the vector DNA causes the adherence of this complex to the chromosomal scaffold in a noncovalent fashion, thus retaining the vector DNA in the nucleus (209). These vectors were reported to replicate somewhat in synchrony with chromosomal DNA once per cell cycle. Maintenance of these vectors within cells is related to the frequency of cell division (208). Such vectors have been reported to persist in cells for at least 2 months under no selective pressure (206,210).

Ultimately, the development of a true mammalian artificial chromosome (MAC) without dependence on viral elements will be the key to obtaining stable episomal replication without dependence on selective pressure. Functional elements in mammalian cells important for maintaining DNA episomally as a minichromosome include a replication origin to promote autonomous replication, telomeres to protect ends of linear DNA and replicate DNA termini, and a centromere to facilitate correct segregation of the construct during mitotic division. Various mammalian chromosomal DNA replication initiation sites have been identified [reviewed in DePamphilis (211)] and found to comprise a 0.5–11 kb primary origin of bidirectional replication (OBR) flanked by an initiation zone of about 6–55 kb. These sequences show characteristics of DNA unwinding, a densely methylated island, attachment sites to the nuclear matrix, and some palindromic sequences.

Vectors utilizing human genomic sequences that promote extrachromosomal vector replication have already been successfully applied as mentioned above. Telomeres that are required for the stability and integrity of the eukaryotic chromosome have been well characterized. In mammalian cells, the telomeric tracts comprise 2–50 kb of tandem TTAGGG repeats. Human centromeres, necessary for proper chromosome segregation at mitosis and meiosis, have been localized cytogenetically as primary constrictions of the chromosomes. They are thought to consist of up to several megabases of highly repetitive DNA belonging to the alpha satellite DNA family (212) and are attached to microtubules (213). Until recently, the functional isolation of the centromere has been a great hurdle in the progress towards the construction of an MAC. The group of Harrington et al. developed the first generation of human artificial microchromosomes (HAC) by creating synthetic alpha satellite arrays ~1 Mb in size (214). They found that such an HAC which is about 6–10 Mb is size is mitotically and cytogenetically stable for up to 6 months in culture in the absence of selective pressure. Nonetheless, the technical challenge of assembling a mammalian artificial chromosome is still formidable as cloning and manipulating such large constructs are not trivial using conventional bacterial cloning systems, and transfer to mammalian cells is difficult.

V. USE OF LIPOSOMES TO DELIVER VECTORS WITH SELECTABLE MARKERS

Liposome-mediated gene transfer appears to be a safe and noninvasive method of DNA delivery into cells. Since high efficiency and stable expression have not yet been achieved using liposomal methods, the use of the human *MDR*1 gene as a selectable marker may allow for the selection and enrichment of the recipient cells and may be useful in the future for the long-term maintenance of the cationic liposome:DNA complex.

Previous studies in our laboratory have shown that a liposomal delivery system can mediate successful *MDR*1 transfection of mouse bone marrow cells and in vivo expression of functional P-gp in hematopoietic cells (97). The introduction via liposomes into hematopoietic cells of an *MDR*1 gene driven by Harvey murine sarcoma virus long-terminal repeat sequences (Ha-MSV-LTR) was achieved either ''directly'' by intravenous administration into mice, or ''indirectly'' by adoptive transplantation of previously in vitro-transfected bone marrow cells. In these studies, using a cationic liposome complex consisting of dioctadecylamidoglycyl spermidine (DOGS) and dioleoylphosphosphatidyl ethanolamine (DOPE), *MDR*1 transfection was detected in up to 30% of unselected and 66% of vincristine preselected murine bone marrow cells as demonstrated by drug resistance in an in vitro, colony-forming unit assay. Although transfection into human bone marrow cells is likely to be much less efficient, the potential of obtaining drug-selectable mouse bone marrow progenitor cells after gene transfer using such a liposome delivery system may eventually make it possible to protect cancer patients undergoing chemotherapy from bone marrow toxicity of anticancer drugs.

Liposome-mediated gene transfer can also be used for in vivo delivery of Adeno-Associated-Virus (AAV)-*MDR*1-based vectors. Recently, drug-selected coexpression of both P-gp and glucocerebrosidase (GC) was achieved with an AAV vector containing the *MDR*1-IRES-GC fusion delivered to NIH 3T3 cells by lipofection (96). Moreover, a single intravenous injection of this bicistronic vector complexed with cationic liposomes into recipient mice allowed detection of GC and *MDR*1 sequences by PCR in all organs tested 7 weeks later.

For nonintegrating DNA vectors such as EBV-based systems (see Section IV) and the AAV system (96), liposome-based gene delivery usually results in transient transgene expression due to the episomal nature of the transfected plasmid and loss of the plasmid when the cells proliferate (215,216). Use of a selectable marker such as *MDR*1 may make it possible to maintain nonintegrated episomal forms in proliferating cells (see Section IV). Since only cells carrying such episomal *MDR*1-based vectors would survive the selection, this advantage should be useful for gene therapy with episomal *MDR*1 vectors in vivo. Combining liposomes with AAV- or EBV-based vectors and *MDR*1 as a selectable marker may make it possible to expand the population of expressing cells by *MDR*1-drug selection.

We are developing a gene therapy model to treat Fabry disease (123) using intravenous injections of a pHa-aGal-IRES-MDR bicistronic vector complexed to cationic liposomes into α-galactosidase A deficient mice (T. Shoshani and M. M. Gottesman, unpublished results). Both human α-Gal and *MDR*1 were detectable in the lungs of the recipient Fabry mice by Southern blot analysis 7 days after injection. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of total RNA extracted from the kidneys of recipient Fabry mice showed the presence of both human α-Gal and *MDR*1 mRNA. The expression in the kidneys was specific to the α−galactosidase-A-deficient mice, where renal tubule cells may be damaged by an accumulation of glycosphingolipids. In situ hybridization analysis localized the mRNA expression to the renal distal tubule epithelial cells. Higher RNA expression was obtained in Fabry mice that were injected 3 times every third day. The repeated administration is tolerated by the recipient mice and no toxic effects were obtained. It remains to be determined whether selection in vivo will allow expansion of cell populations expressing human α-Gal by repeated administration of cytotoxic *MDR*1 substrates.

VI. ENGINEERING MDR VECTORS TO IMPROVE EFFICIENCY OF DRUG SELECTION

One of the goals of gene therapy is to modify cells genetically such that they can supply a useful or necessary function to the cell (3). One of the most promising applications of the *MDR*1 gene in therapeutic vectors as a selectable marker in vivo is the protection of bone marrow cells during intensive chemotherapy. During chemotherapy, the *MDR*1 gene is transduced or transfected into drug-sensitive bone marrow cells and selected for by exposure to MDR agents. The untransfected/untransduced cells will necessarily be killed and those containing the *MDR*1 gene will expand. The efficacy of this therapy depends on the interaction between P-gp and the selecting agent employed. Thus, it is important to be able to distinguish between the endogenous P-gp and the exogenously introduced molecule. Furthermore, it obviously would be beneficial to create a P-gp molecule that would confer very high levels of resistance to certain drugs, giving an advantage to transduced cells/tissues compared to wild-type P-gp. Studies of a number of mutations made in P-glycoprotein have suggested that it should be possible to construct mutant ''designer'' transporters useful for *MDR*1-based gene therapy.

One of the hallmark characteristics of the multidrug transporter is its extremely broad substrate specificity. Over the past several years, the identification of specific domains and amino acid residues involved in substrate recognition has contributed to our present understanding of the mechanism of action of P-gp. The major sites of interaction have been shown to reside in transmembrane domains (TM) 5 and 6 in the N-terminal half of the protein and in TMs 11 and 12 in the C-terminal half and the loops that conjoin them (217–221). For the purposes of chemoprotection, the design of a P-gp that has increased resistance to chemotherapeutic agents compared to the endogenous P-gp would be most useful because increased doses of the agent could be administered without harming the bone marrow cells expressing the exogenous P-gp molecule. To date, a number of these types of mutations have been described.

Mutations in TM domains of P-gps from both rodent and human have demonstrated significant alterations in substrate specificity (3,222). An F338A mutation in hamster P-gp enhances resistance to vincristine, colchicine, and daunorubicin but has little impact on resistance to actinomycin D (223,224). An F339P mutation in the same molecule only increases actinomycin D resistance. However, the double F338A/F339P mutant demonstrates an increased level of resistance to actinomycin D and vincristine but a lowered level of resistance to colchicine and daunorubicin (223,224). Of these mutants, the F338A may prove most useful because it confers increased resistance to a wider range of chemotherapeutic agents. In human P-gp, however, a homologous mutation at F335 confers greater resistance to colchicine and doxorubicin but causes a severe reduction in resistance to vinblastine and actinomycin D (225,226) . Additionally, cells expressing a Val->Ala mutation at position 338 also exhibit preferential resistance to colchicine and doxorubicin but are severely impaired for vinblastine (226). Resistance to actinomycin D, however, is unaffected. Alanine scanning of TM 11 in mouse P-gp encoded by *mdr*1a revealed that two mutants, M944A and F940A, show an increase in resistance to doxorubicin and colchicine while maintaining wild-type levels of resistance to vinblastine and actinomycin D (227). For certain treatment protocols, it is conceivable that increased resistance to certain agents would be desirable, and the reduction in levels of resistance to other compounds would not be problematic, especially if a well-defined chemotherapy regimen was being employed.

Although the majority of residues that increase resistance to various chemotherapeutic agents reside in the TM domains, a number of residues in the putative cytoplasmic loops also have been implicated in defining drug resistance profiles for cytotoxic drugs. The best characterized of these mutations is the G185V mutant that confers an increased resistance to colchicine and etoposide but decreased resistance to actinomycin D, vinblastine, doxorubicin, vincristine, and taxol

(228–231). Interestingly, and perhaps relevant clinically, when this mutation is made in conjunction with an Asn->Ser mutation at residue 183, increased resistance to actinomycin D, vinblastine, and doxorubicin is achieved without loss of the increase in colchicine resistance (229). Mutations of Gly-141, 187, 288, 812, or 830 to Val in human P-gp increase the relative resistance of NIH3T3 cells to colchicine and doxorubicin but do not alter resistance to vinblastine (232). Only the mutations at positions 187, 288, and 830 confer decreased resistance to actinomycin D to cells in culture.

Due to its broad substrate specificity, P-gp not only interacts with chemotherapeutic compounds but also with reversing agents and inhibitors. In combination chemotherapies, reversing agents increase the efficacy of cytotoxic agents in *MDR*1-expressing cancers. Two of the most potent reversing agents currently in use or in clinical trials are cyclosporin A and its nonimmunosuppressive analog PSC833. Recently, a number of mutants have been described that affect sensitivity to these agents. Cells expressing a human P-gp containing a deletion at Phe335 or Phe334 are substantially resistant to cyclosporin A and PSC-833 [(233), Hrycyna, C.A., Pastan, I., and Gottesman, M.M., unpublished data]. A similar phenotype has been observed for a transporter containing 5 mutations in the region including TM 5 and TM6, namely Ile299Met, Thr319Ser, Leu322Ile, Gly324Lys, and Ser351Asn (234). Additionally, in hamster P-gp, the substitution of an alanine at position 339 with proline results in a transporter that confers lowered sensitivity to cyclosporin A (224). From these studies, it appears that TM6 plays an important role in the recognition of cyclosporin A and its analogs. The decreased sensitivity to these reversing agents observed in cells expressing the TM6 mutations could help protect bone marrow stem cells transduced with the mutant *MDR*1 gene from the toxic effects of chemotherapy given with reversing agents to sensitize *MDR*1-expressing tumors.

The *cis* and *trans* isomers of flupentixol, a dopamine receptor antagonist, have also been shown to inhibit drug transport and reverse drug resistance mediated by P-gp (235,236). The substitution of a single phenylalanine residue at position 983 with alanine (F983A) in TM 12 affects inhibition of P-gp-mediated drug transport by both isomers of flupentixol (59,60,237). Both isomers were found to be less effective at reversing P-gp mediated drug transport of daunorubicin and bisantrene. However, the inhibitory effects of other reversing agents such as cyclosporin A were not affected. The reduced sensitivity of the F983A mutant to this compound coupled to the apparent lack of clinical toxicity of (*trans*)-flupentixol (235), suggests that this mutant may be useful in combining *MDR*1 gene therapy with chemotherapy including *trans*-flupentixol as a chemosensitizer. This approach, in theory, should allow for effective treatment at lower doses of chemotherapeutic agents while maintaining bone marrow protection.

The use of *MDR*1 gene therapy in bone marrow chemoprotection protocols has undergone preliminary analysis in clinical trials (89,90,238). Results indicate a low efficiency of marking bone marrow cells using retroviral vectors, but some selective advantage manifested as an increased percentage of positive cells after chemotherapy (89). In the future, with the generation of higher resolution structures of human P-gp, it should be feasible to model and synthesize new, more effective cytotoxic drugs or modulators capable of blocking P-gp function clinically. However, until that time, the analysis of spontaneously occurring or engineered mutants, coupled to our knowledge of the current battery of anticancer and reversing agents, offers an opportunity to begin designing second-generation vectors for use in these trials.

VII. CONCLUSIONS AND FUTURE PROSPECTS

We have argued in this review that drug-selectable marker genes may be helpful for gene therapy in two ways: first, to protect bone marrow progenitor cells (and other sensitive cells) from the cytotoxicity of anticancer drugs, thereby allowing safe chemotherapeutic treatment at reduced risk of severe side effects, and second, to enrich the expression of otherwise nonselectable genes in drug-sensitive cells to overcome low or unstable gene expression in vivo. Given the current instability of expression of genes from existing vectors, especially episomal vectors, such selectable markers may be an essential component of gene therapy protocols.

We are still in the early stages of vector development, and until transduction efficiencies into human tissues such as bone marrow are improved and shown to be safe, long-term human gene therapy will not be feasible. The combination of more efficient gene transfer targeted vector systems, and effective, relatively nontoxic selection systems to maintain gene expression may make long-term correction of human genetic defects feasible and safe.

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22

Ligand-dependent Transcription Switches and Their Potential for Gene Therapy

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I. TRANSCRIPTIONAL REGULATION IN GENE THERAPY

The potential advantage offered by gene therapy over conventional medicine for the treatment of several diseases is unquestionable. It is nonetheless also clear that efficacy, and more importantly safety, remain the main issues to be solved before this technology can be adopted routinely as a standard therapeutic practice (1,2). At the time of writing this article, beginning in 2003, clinical efficacy of gene transfer has been convincingly demonstrated only for Severe Combined Immune Deficiency (SCID). In contrast, safety concerns have been raised as a consequence of adverse side effects in several clinical trials (3–8). Therefore, the major challenge still remains the development of vectors characterized by maximum transduction efficiency combined with minimal toxicity (9,10). In current years the continuous application of ''good basic science'' to the gene therapy field has been directed at fulfilling these two main goals. With the decline of the initial concept of a ''universal vector'' for all diseases, the emerging scenario is to generate a large repertoire of safe vectors, each suited to target a given tissue or cell type, or tailored to treat a specific clinical condition. (11–13). Based on these premises, any work directed at developing systems, which allow to control and finely tune the expression of therapeutic gene(s), must be taken into serious consideration.

Until now, clinical trials of somatic gene therapy have made exclusive use of constructs where transgenes are delivered under the control of potent and constitutively active promoters. In these applications the risk of toxic effects caused by overproduction of the therapeutic proteins was negligible for a variety of reasons, related partly to the features of the vector used and in part to the nature of the target disease. In some cases short-term transgene expression was expected because the vector, for example 1st generation adenovirus, was not capable per se of long-term gene expression (2). In others, such as naked DNA delivered to muscle, it was known that gene transfer was not efficient enough to elicit too high levels of the transgene product (14). When cancer or other acute diseases were targeted, prolonged treatment was not required, and the main goal was to maximize gene transcription and protein production for a short period of time (15). Finally, in clinical trials of chronic diseases, where long-term gene expression was required, gene therapy was directed at curing clinical conditions in which the expressed proteins had large therapeutic indexes (TIs). Examples of this are coagulation factor IX for Hemophilia B, IL-2 receptor gamma-chain or adenosine deaminase for SCID, and CFTR for Cystic Fibrosis (16–18).

Constitutive promoters, however, have limited applications in gene therapy. The main reason is that they cannot be used for the delivery of proteins with small TI. In practical terms, several diseases could benefit from the delivery of genes whose activity must be kept within a narrow therapeutic window. Good examples are disease caused by protein hormone deficiencies, such as anemia or pituitary dwarfism, or those that require treatment with soluble receptors, cytokines and antibodies (19). These clinical conditions are currently treated by repeated administration of recombinant proteins, but less frequent or one-time delivery of the gene coding for the therapeutic protein would indeed represent a more cost-effective and successful approach (14). For these cases, regulating transgene expression would be crucial to maintaining the circulating levels of the protein within a well-defined therapeutic window, thereby preventing toxicity. Regulated gene tran-

scription would also allow therapy to be modulated in response to disease evolution, which varies greatly from patient to patient, and in the individual response to the therapy itself. Finally, the possibility to terminate and resume therapy (i.e., stop and restart transgene production) at will, would not only allow therapy to be halted in the presence of adverse side-effects, but would also enhance the flexibility of a gene-based approach by enabling combinations with conventional therapeutic modalities.

In addition, it has to be taken into account that vector improvements will probably increase efficiency and longevity of gene transfer over the next year. As a consequence, the expression levels of proteins with large therapeutic indexes will also need to be controlled. In the long term, therefore, it is not premature to think that regulating therapeutic gene expression will become an indispensable mechanism in broadening the application range of gene therapy and increasing both clinical efficacy and safety of the majority applications.

A. Endogenous Regulatory Systems

To be used in the context of human gene therapy, a transcription regulation system must precisely modulate target gene expression in response to administration or withdrawal of a specific external stimulus. Initial attempts to generate regulated gene expression systems were mainly based on using endogenous promoters and enhancer elements specifically responsive to environmental stimuli such as metal ions, heat, and oxygen tension (20). A detailed description of these systems is beyond the scope of this chapter, but it is worth remarking that these types of regulatory systems have severe drawbacks. The most important is that the inducer stimuli heavily interfere with the regulatory networks of the host and therefore display pleiotropic effects. Moreover, these systems display a relatively high basal activity in the uninduced state, promote only modest levels of induction, and frequently lose responsiveness to the inducer over time (20). These limitations obviously preclude the use of endogenous control systems in human gene therapy, and have been gradually superseded by more efficient approaches based on exogenous regulators.

B. Ligand-dependent Regulatory Systems

Over the past decade, several artificial systems have been developed that enable regulable expression of the desired transgene in response to a small molecule ligand (21). In general, these systems are based on two components. The first is a chimeric transcription factor obtained by fusing a DNA-binding domain (DBD), (which usually does not bind endogenous cellular sequences), a transcription activation domain (AD), and a domain that interacts with a small molecular weight compound that acts as inducer drug. The second component is an artificial promoter consisting of multimeric binding sites for the DBD followed by a minimal promoter sequence containing a TATA box. The chimeric transcription factor is recruited to (or in some cases released from) the specific target promoter upon interaction with the exogenously added drug: transcription of a transgene cloned downstream of this promoter can thus be modulated in vivo by systemic delivery or withdrawal of the inducer drug (Fig. 1).

To be suitable for human gene therapy, the ideal pharmacologically regulated system should fulfill several criteria listed below:

1. ON switches vs. OFF switches: the ligand should activate (ON switches) rather than inhibit (OFF switches) activity of the chimeric transcription factor. In practice, off switches are unlikely candidates for human gene therapy because of two major drawbacks: in order to fully repress an off-switch system, all intracellular molecules of the chimeric activators must theoretically be bound to the drug. This implies that prolonged exposure of patients to high doses of the drug would be required to silence the system in vivo, thus increasing the probability of drug-related side effects. Secondly, induction kinetics would be poor in the case of off switches and mainly determined by the rate of drug clearance (inactivation of the drug or its removal from body tissues).
2. Specificity: the system should not interfere with endogenous regulatory pathways. This means that the transcription factor should only activate the target promoter and the drug should be devoid of pleiotropic effects.
3. Bioavailability of the drug: the inducer drug should be orally bioavailable and capable of readily penetrating all tissues, as well as crossing the blood-brain barrier in cases of gene therapy to the CNS.
4. Drug safety: the inducer compound must have a safety profile compatible with prolonged therapeutic use in humans.
5. Reversibility: the system should be fully and rapidly reversible to enable prompt modification of the dosing regimen when required. In relation to this, it is important that the inducer drug is cleared from body tissues within a reasonable amount of time, thus enabling rapid switching from the on- to the off-state upon drug delivery and withdrawal, respectively.
6. Low basal activity: the system should be inactive in the absence of the drug when delivered with any type of vector of both viral and non viral origin. It is important that this tight control should be maintained at all vector doses and in every target tissue.
7. High degree of inducibility: the system should be induced over a wide dose range. In particular, strong induction should be obtained at relatively low (i.e., compatible with therapy in humans) drug concentrations.

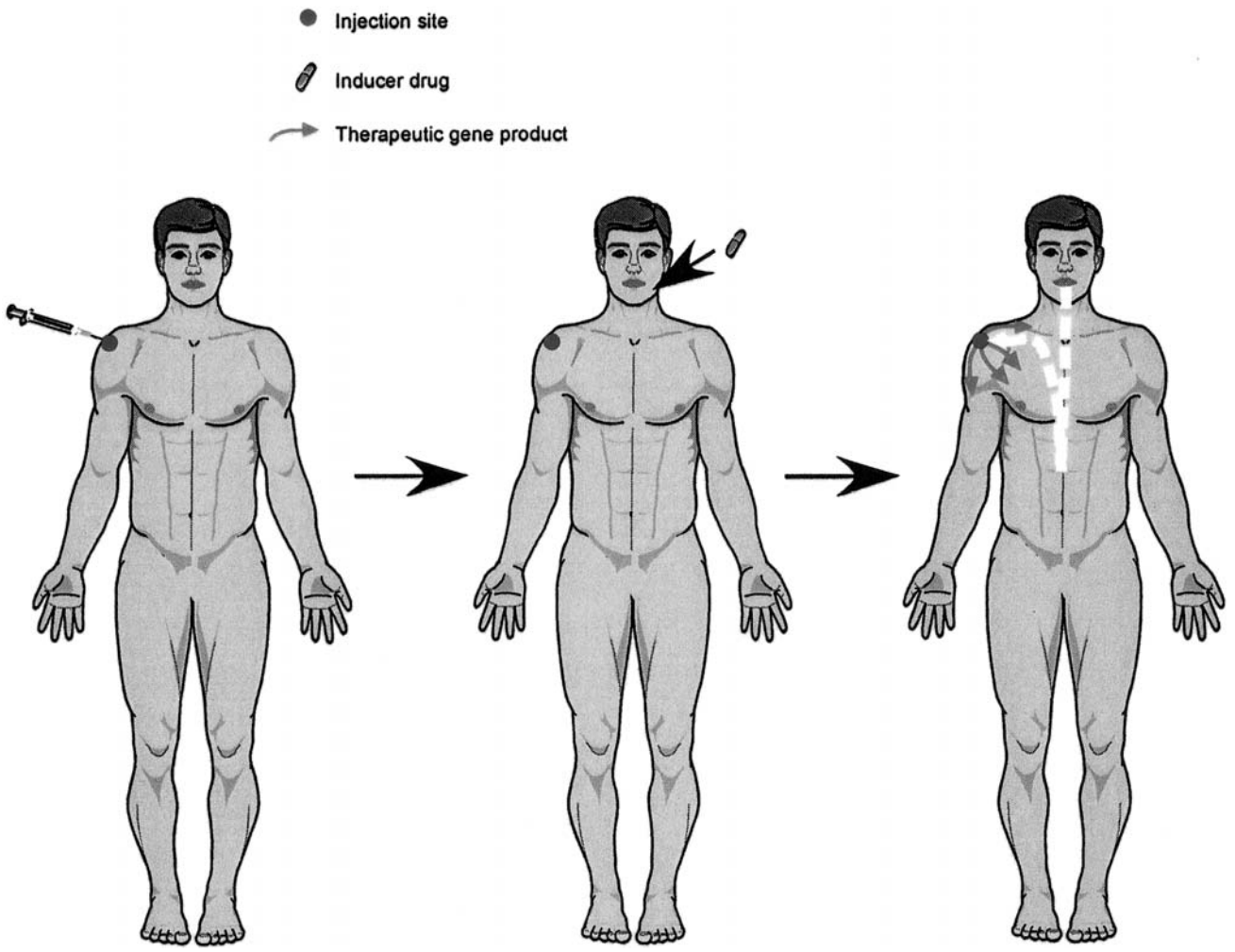


Figure 1 Drug-dependent expression of a therapeutic gene. Local delivery of a ligand-regulated gene results in systemic production of the corresponding therapeutic protein upon drug administration. See the color insert for a color version of this figure.

8. Dose-dependence: a pre requisite for a fine modulation of protein production in vivo is that a precise correlation must exist between drug dosage and target gene expression level.
9. Low immunogenicity: to enable long-term applications, the chimeric transcriptional activator(s) should exhibit a low potential of eliciting an immune response in man.

Several ligand-dependent systems have been described in the past years, but only four of them have been extensively tested in animal models and are currently being refined for use in human applications. These four systems are regulated by a) the tetracycline (Tet) antibiotics or its analog doxycycline (Dox); b) chemical dimerizers such as the immunosuppressants rapamycin and its analogs; c) the synthetic steroid antagonists mifepristone (RU486) and tamoxifen (TAM); and d) insect steroid ecdysone or its analogs. In this chapter we will review in depth ligand-dependent gene regulation with particular emphasis on these four systems, and will discuss to what extent they come close the ideal features outlined above.

II. TETRACYCLINE-DEPENDENT REGULATORY SYSTEM

A. General Principles

The tetracycline-dependent regulatory system is based on the *E. coli* Tn*10*-encoded tetracycline resistant operon, which was adapted to work in eukaryotic cells. Tetracycline (Tc) resistance operon consists of two genes, the resistance gene *TetA*, which codes for a membrane protein that exports invaded Tc

out of the bacterial cell, and the regulator gene *TetR*, which codes for a dimeric DNA-binding protein (22). In the absence of Tc, TetR protein inhibits its own expression as well as expression of TetA by binding to operator sequences (tetO) of the tet operon. Tc or other antibiotics belonging to the same class, such as doxycycline (Dox) prevent this binding (Fig. 2). These antibiotics bind to the TetR and induce allosteric change, which results in releasing the repressor from its target DNA sequence, thus enabling transcription of *TetA* and *TetR* genes (22).

The Tc operon evolved to strongly repress TetA expression in the absence of the antibiotic, but at the same time, to sense very low (i.e., subinhibitory) concentrations of Tc. Therefore, TetR binds to tetO with extremely high affinity (2×10^{11} M^{-1}) and the association constant of Tc to tetR is very high ($K_{Ass} = 3 \times 10^9$ M^{-1}) (22) and references therein]. Based on these properties, the use of elements of the Tc operon to regulate gene expression in eukaryotes was considered an attractive possibility.

Initial studies in plants demonstrated that TetR was able to repress activity of a plant promoter containing three tetO sequences in close proximity to the TATA box (23). In this configuration, binding of TetR interfered with initiation of transcription. As in bacteria, repression was reversible by Tc: upon adding the antibiotic, the TetR was released from DNA and transgene expression was induced (23). However, several lines of evidence indicate that the repressing mechanisms active in bacteria are not appropriate for regulating promoter activity in eukaryotes. In fact, a repressor-binding site, such as tetO, should be efficiently (i.e., almost fully) occupied by the repressor to adequately inhibit transcription: this implies that when tight repression is required, the repressor itself should be expressed at high and possibly toxic levels within the target cells. Moreover, constitutively high intracellular concentrations of the repressor may severely limit the inducibility of the system (24). Finally, studies in cells have demonstrated that efficient silencing of eukaryotic promoters requires proper positioning of tetO according to rules that may vary in accordance to the target promoter (25).

Regulatory systems based on ligand-regulated activators have less severe requirements: in the simplest case, the target promoter is constitutively silent and becomes active only when bound by the cognate activator. Notably, overexpression of the activator is not necessary, provided it is present above a certain threshold level. These considerations prompted H. Bujard and colleagues to convert the TetR into a positively acting factor and use it to regulate gene expression in eukaryotic cells (26).

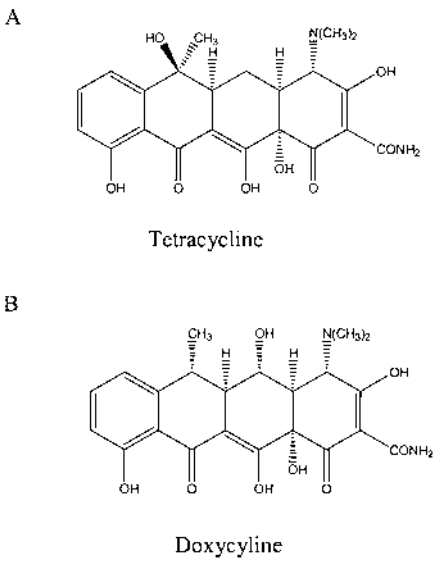


Figure 2 Chemical structure of tetracycline and doxycycline, inducers of the Tet-system. (A) Tetracycline effectively stimulates tTA (Tet-OFF system) but not rtTA (Tet-ON system). (B) Doxycycline activates both Tet-ON and Tet-OFF systems.

B. Transcription Factors

TetR was converted to a transcriptional transactivator, called tTA, by fusing the VP16 transactivation domain of *H. simplex* virus to the C-terminus of TetR (26). tTA retained the DNA-binding and ligand-binding properties of TetR: it was prevented from binding to tetO by Tc or its derivatives, such as doxycycline (Dox), a most potent effector for both TetR and tTA. tTA proved capable of activating expression from tetO-containing promoters in a tetracycline-dependent manner in eukaryotic cells (26). In this ''Tet-OFF'' configuration, therefore, transcription is stimulated by drug withdrawal (Fig. 3). tTA retained an exquisite responsiveness to the effector molecule, in that it was completely inactivated by Dox concentrations as low as 20 ng/ml (26,27). Since its development, the Tet-OFF system has become one of the most widely used systems for modulating gene expression in cells and transgenic animals (28).

As outlined in the introduction, however, a system in which gene expression is induced by drug administration rather than by its withdrawal is highly preferable in the vast majority of gene therapy applications. To generate a Tc-inducible switch, the allosteric behavior of TetR was reversed. By combining chemical mutagenesis and genetic selection in *E. coli*, a mutant form of TetR was identified that carries four amino acid substitutions and is able to bind tetO only in the presence of tetracycline. Fusion of this mutated TetR to VP16 produced a reverse tTA (rtTA), capable of stimulating transcription only upon adding ligand (Tet-ON system, Fig. 4) (27). However, the same mutations that reversed the phenotype of tTR also affected its affinity for the inducer drug. In fact, rtTA was not induced by Tc and was about 100-fold less responsive to Dox than tTA (27). As a consequence, maximum activation of rtTA is achieved at Dox concentrations as high as 1–3 μg/ml, which is only about 5-fold lower than the toxic dosage for mammalian cells (29). Moreover, rtTA displayed a significant basal activity that was mainly due to a residual binding of rtTA to

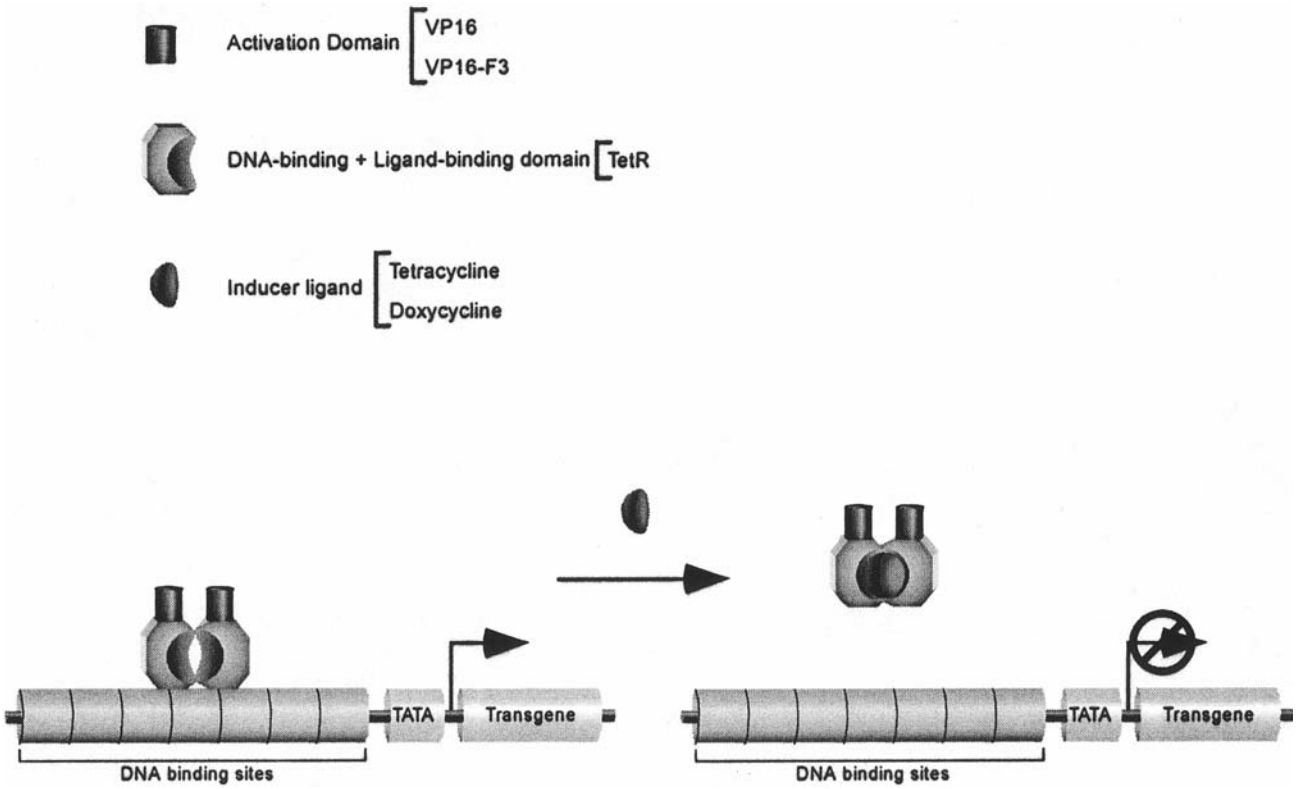


Figure 3 tTA-based, Tet-OFF system. tTA consists of the tetR fused to VP16 or VP16-derived activation domain. In the absence of the inducer drug, tTA binds and activates a promoter consisting of a minimal TATA box containing promoter located downstream of seven tet operator (tetO) sites. Upon addition of Tc or Dox, tTA is released from DNA and the promoter becomes inactive. For the sake of simplicity, only one molecule of tTA is represented.

tetO in the absence of the inducer Drug (28–30). Both factors limit the potential of tTA for gene therapy applications (see below).

More recently, improved versions of rtTA have been isolated by functional selection in yeast (30). These novel transactivators, called rtTA2^S-S2 and rtTA2^S-M2, carry different mutations compared to rtTA but retain Dox-dependency and display considerably lower activity in the off state (30–33). These latest versions of the rtTA also carry an optimized activation domain (VP16-F3) consisting of three tandem repeats of a 12 amino acid peptide derived from the VP16 activation domain (34). Finally, the cDNAs for these novel transactivators have been optimized for expression in human cells. Because of their lower leakiness, both rtTA2^S-S2 and rtTA2^S-M2 were better inducers than rtTA and enabled hundreds-fold stimulation of gene expression in transiently and stably transfected cells (30–32). rtTA2^S-S2 displays the lower uninduced activity between these two novel versions, thus enabling more stringent control. rtTA2^S-M2 is more responsive to Dox, as it is about 10-fold more sensitive to Dox than rtTA and is fully induced by Dox concentrations as low as 100–200 ng/ml (30,33).

A modified version of the tet system has been used to repress transcription in a ligand-dependent manner. Dox-dependent silencing was achieved by fusing the transrepressor KRAB (Kruppel-associated box) domain of the human Kid protein to the tetR (35,36). In the absence of Dox, this transrepressor, called tTS, was able to silence a CMV promoter engineered to contain seven tetO sequences in its 5′ region (36). In the presence of the effector molecule, tTS is released from DNA and gene expression is induced. For the reasons outlined above, systems based on repression are probably not appropriate for gene therapy.

More interestingly, tTS has now become a standard technology in combination with rtTA to repress basal activity in uninduced conditions, thus widening the regulatory window of the Tet-ON system. A tTS unable to heterodimerize with rtTA was designed, which contains a dimerization domain of different class specificity compared to rtTA (37,38). As indicated in Fig. 5, in the absence of Dox, tTS binds to *tetO* and inhibits basal transcription: as Dox is added, tTS dissociates from the target DNA while rtTA becomes active and triggers transcription (37,38). Coexpressing tTS and rtTA (rtTA/tTS system) reduces the basal expression to almost un-

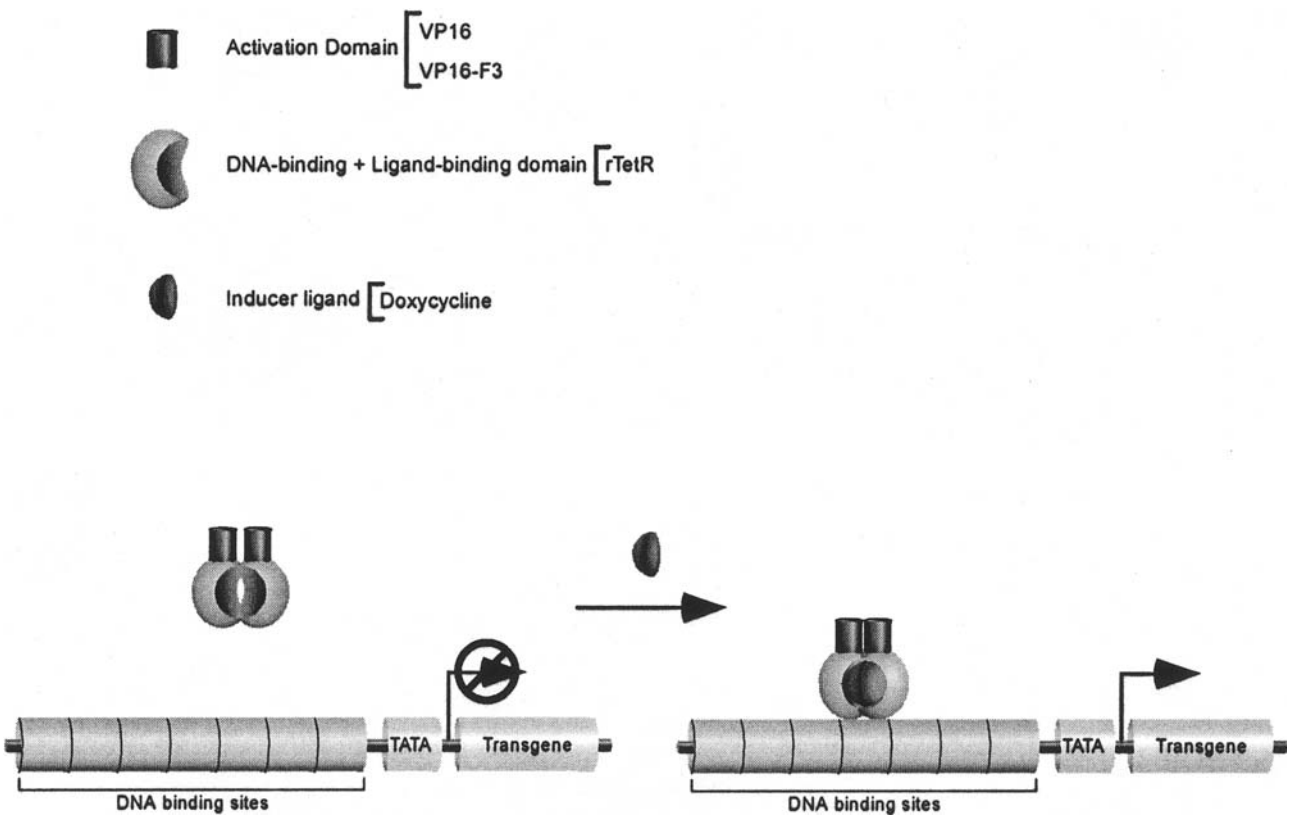


Figure 4 rtTA-based, Tet-ON system. rtTA consists of rTetR fused to an activation domain, usually derived from VP16. rtTA is inactive in the absence of the drug: following Dox administration, it binds and activates the target promoter. See text for additional details.

detectable levels in vivo (39–43). Despite these attractive features, one drawback of the rtTA/tTS system is that it is fully active only at high (μg/ml) Dox concentrations, because of the low responsiveness of rtTA to Dox (33,37,44). From this viewpoint, the use of the most advanced version of rtTA in combination with tTS proved useful. The properties of rtTA/tTS, rtTA2^S-S2/tTS, and rtTA2^S-M2/tTS combined systems have been compared in a recent study (33). Both rtTA2^S-S2/tTS and rtTA2^S-M2/tTS displayed a lower baseline activity and a 10-fold expanded dynamic range of induction than rtTA/tTS, with thousands-fold induction measured in Dox-treated cells in the absence of detectable leakiness. Moreover, the rtTA2^S-M2/tTS system displayed the highest Dox sensitivity, as it became fully active at concentrations as low as 100–200 ng/ml of Dox (33). Of course, the greater Dox-responsiveness of rtTA2^S-M2/tTS makes this system more suitable for gene therapy applications in humans.

C. Tet-responsive Promoter

The Tet-responsive promoter in its more classical configuration ($P_{hCMV*-1}$, also called P_{tet-1}) consists of a minimal version of the human cytomegalovirus immediate-early promoter fused 5′ to a heptameric tetO repeat (tetO7) (29). In this configuration, the promoter may display a relatively high leakiness because of the intrinsic basal activity of the CMV minimal promoter (29). The use of different minimal promoter sequences, such as a modified mouse mammary tumor virus (MMTV) promoter or a core promoter derived from the plant viral 35S promoter, decreases the basal activity of the Tet-responsive promoter by one order of magnitude (45,46). However, such promoters are also considerably less potent than $P_{hCMV*-1}$ upon induction with Dox: therefore, it may be particularly appropriate to use them in those cases where stringency of control is the most critical factor. It is worth mentioning that the leakiness of $P_{hCMV*-1}$ is effectively suppressed by tTS also when coexpressed with the various activators (see above).

The heptameric version of the tetO was initially evaluated as the most potent operator, and therefore little or no effort was dedicated to modifying this architecture (26). In point of fact, the heptameric sequence is the standard target sequence for tTA, tTS, and the various versions of rtTA. It has been recently reported that IFNα-stimulated response elements (ISREs) are located in the linker regions between the heptam-

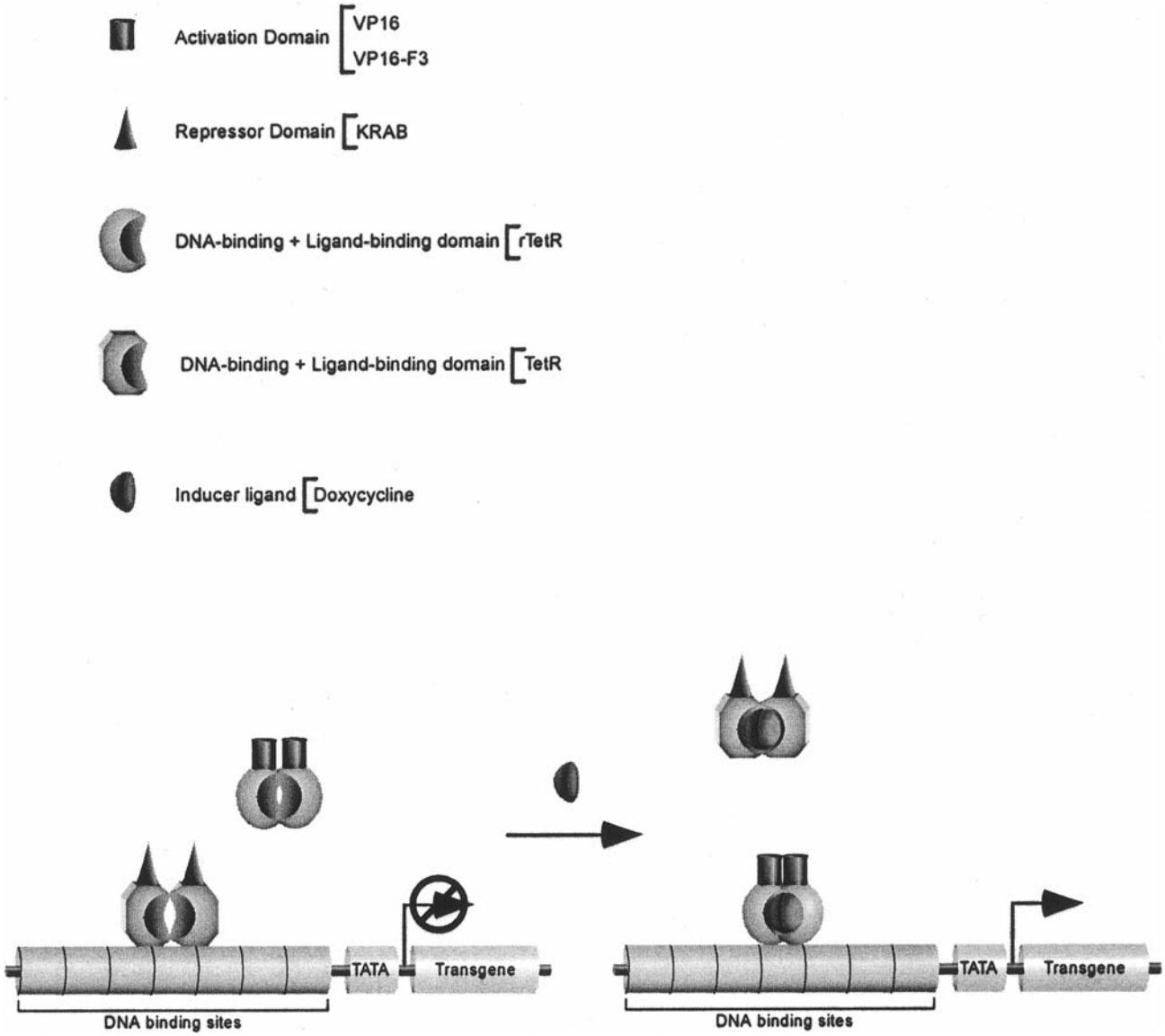


Figure 5 rtTA/tTS-based system, Tet-ON system. rtTA consists of rTetR fused to the VP16 activation domain and tTS consists of TetR fused to the KRAB repressor domain. rtTA and tTR recognize the same DNA sequence but cannot dimerize because they carry dimerization domains of different class specificity. In the absence of Dox, tTS binds and represses the target promoter. Following drug administration, tTS is released from DNA while rtTA interacts with the target promoter, thus triggering gene expression.

eric tetO sequences (47). Consequently, IFNα can stimulate tet promoter activity, thus interfering with the stringency of gene control (47). To avoid undesired induction, these ISREs would probably have to be deleted before this system can be used in humans.

D. Tet System for Gene Therapy Applications

The various versions of the Tet systems have been delivered in animal models by a variety of vectors and in several different tissues to regulate expression of reporter as well as therapeutic genes (29).

The tTA system has been and continues to be used to regulate transgene transcription in vivo in a variety of animal models (48–54). However, because of the unfavorable properties of an OFF-system in vivo, these reports can be considered as interesting proof-of-concept studies, which are nonetheless unlikely to move from the preclinical to the clinical setting.

In contrast, results obtained with the various configurations of the Tet-ON system are of greater interest and forecast future

outcomes in humans. A few reports, listed in Table 1, have indeed demonstrated that the original rtTA-based system, delivered in mice and nonhuman primates using both viral and nonviral vectors, can be used for long-term regulation of gene expression in a Dox-dependent manner (55–58). However, a certain leakiness was observed, especially in those cases where rtTA was used for modulating expression of high-potency protein hormones, such as erythropoietin (Epo), the major regulator of erythropoiesis in mammals (59). In this case, even the low amount of protein produced in the uninduced state stimulated a hematocrit (Hct) increase in untreated mice (31,55,57).

In vitro and in vivo leakiness was more pronounced in those cases where the rtTA cDNA was cloned downstream of strong enhancer/promoter elements and inserted with the tet-responsive gene into the same vector (55,60). This type of configuration probably results in very high intracellular concentrations of rtTA, which may thus unspecifically activate transcription. Moreover, the basal activity may be increased by the interference between the regulatory elements driving rtTA expression and the intrinsically ''leaky'' $P_{hCMV*-1}$ promoter. As a matter of fact, more efficient control could be achieved by splitting the system into two vectors, thus allowing to cells to be transduced with substoichiometric amount of transactivator with respect to the regulated gene (56,57,61).

As expected on the basis of in vitro results, the novel versions of rtTA enabled tighter control when used in vivo (Table 1). Better results were obtained with the less leaky $rtTA2^S$-S2. In a mouse model of hepatitis, intrahepatic delivery of a gutless-Ad vector carrying an $rtTA2^S$-S2-regulated IFNα protected mice from the disease: protection was only observed in mice treated with Dox, which stimulated IFNα production in a Dox-dose dependent manner (62). $rtTA2^S$-S2 also enabled tight control of Epo gene when delivered intramuscularly by electroinjection as plasmid DNA (31). Epo production and Hct levels could be modulated up to 300 days postinjection in response to Dox delivery and withdrawal, in the absence of any background expression in untreated mice. Notably, this result was achieved by using a single plasmid containing a CMV-$rtTA2^S$-S2 expression cassette and the tet-responsive Epo gene (31). In the same experimental setting, $rtTA2^S$-M2 displayed a certain degree of leakiness, which was, however, much lower than that of rtTA (31). The leakiness of $rtTA2^S$-M2 was also evident in another study, in which a gutless-Ad vector containing $rtTA2^S$-M2-regulated secreted alkaline phosphatase (SEAP) gene was delivered into mouse muscles (63). In vivo experiments thus confirmed that $rtTA2^S$-S2 represented a major improvement over rtTA in terms of basal activity. Moreover, Dox-dose response experiments demonstrated that the system is activated in mice by oral dosages of Dox comparable to those normally used in clinical practice in humans (31). Nonetheless, vector-dose response experiments demonstrated that the tightness of control is partially lost at higher vector doses, thus imposing an upper limit to the amount of vector that can be delivered (31,33,62). Use of this activator may be appropriate in those cases in which a certain degree of basal expression level is acceptable.

Coexpression of tTS has been adopted as an alternative strategy to reduce the basal activity of rtTA following in vivo delivery. For historical reasons, initial studies involved the use of tTA with rtTA (Table 1). In a first study, the system

Table 1 Tet-on System: In Vivo Studies in Animal Models of Gene Therapy

Transactivator	Transgene[a]	Vector	Tissue	Species	Refs.
rtTA	GFP	Ad	brain	rat	56
rtTA	Epo	AAV	muscle	mouse	55
rtTA	Epo	Plasmid DNA	muscle	mouse	57
rtTA	GFP, GH	AAV	retina	mouse	61
rtTA	Epo	AAV	muscle	monkey	58
rtTA	Epo	Plasmid DNA	muscle	mouse	31
$rtTA2^S$-S2	IFNα	HD-Ad	liver	mouse	62
$rtTA2^S$-S2	Epo	Plasmid DNA	muscle	mouse	31
$rtTA2^S$-M2	Epo	Plasmid DNA	muscle	mouse	31
$rtTA2^S$-M2	SEAP	HD-Ad	muscle	mouse	63
$rtTA2^S$-M2	SEAP	Plas DNA, Ad	muscle	monkey	69
rtTA/tTS	GFP	AAV	retina	mouse	39
rtTA/tTS	Leptin	AAV	hypothalamus	mouse	43
rtTA/tTS	Epo	AAV	muscle	mouse	40
rtTA/tTS	IL10	Plasmid DNA	muscle	mouse	42
$rtTA2^s$-M2/tTS	SEAP	HD-Ad	muscle	mouse	63
$rtTA2^s$-M2/tTS	Epo, SEAP	Plasmid DNA	muscle	mouse, monkey	33

[a] GFP = Green Fluorescent Protein; Epo = erythropoietin; GH = Growth Hormone; IFNα = Interferon α; SEAP = Secreted Alkaline Phosphatase; IL10 = Interleukin 10.

was split into two AAV vectors: a regulatory vector expressing in a constitutive manner rtTA and tTS, and a reporter vector containing a tet-responsive Green Fluorescent Protein (GFP) cDNA. The two vectors were coinjected subretinally in mice at a 1:1 ratio: GFP expression was only detected in Dox-treated animals, with no evidence of basal expression in untreated mice (39). Similarly, a binary AAV vector system injected into rat hypothalamus was used to regulate leptin expression in a Dox-dependent manner (43).

However, more sophisticated vector architecture was required to tightly control Epo expression using a binary AAV system. Because of its highly specific activity, Epo expression in the uninduced state was further decreased. This was achieved by modifying the architecture of the reporter AAV vector, which in this case contained both Epo and tTS under the control of the $P_{hCMV^{*}\text{-}1}$ tet-responsive promoter (40). Intramuscular coinjection of this vector with the rAAV expressing rtTA and tTS allowed a stringent control over an 8-month period, with the untreated mice showing normal Hct levels for the whole duration of the experiment. Repeated cycles of induction were successfully performed, indicating that the silencer did not interfere with reinducibility of the system (40). The fact that leakiness is strongly dependent on vector structure is also suggested by a recent study reporting an efficient but not absolutely tight control of IL-10 expression upon intramuscular electroinjection of plasmid DNA (42). Also in this case a combination of two-vector system was used, but the vector containing the inducible IL-10 also expressed rtTA, with tTS being expressed from the other plasmid vector (42). Results with the rtTA/tTS system thus demonstrated that tTS significantly reduced the basal activity of the system in vivo, provided that vector architecture is optimized.

In a further advanced and more recent version of the system, the repressing activity of tTS has been combined with the reduced leakiness and enhanced Dox-responsiveness of the $rtTA2^{S}$-M2 (Table 1). Bicistronic cassettes have been constructed that coexpress tTS with the novel $rtTA2^{S}$-M2 activator via an internal ribosome entry site. When tested in transient transfection, this cassette mediated 10^{4}-fold induction of reporter gene expression in a strictly Dox-dependent manner and with no detectable leakiness (33). When used in the context of gutless-Ad vectors or of plasmid DNA delivered into mouse muscles, the $rtTA2^{S}$-M2/tTS system mediated thousands-fold induction of SEAP expression, with no basal activity in uninduced mice (33,60). Maximum induced levels were comparable to those achieved with vectors expressing SEAP under the control of the CMV enhancer/promoter element mice, confirming the efficiency of the system (33,60). The same $rtTA2^{S}$-M2/tTS switch delivered as plasmid DNA in mouse muscles mediated stringent modulation of Epo gene expression over a 1-year period (33). With both adenoviral and plasmid vector, transgene expression was strictly dependent on Dox administration and completely extinguished upon Dox withdrawal: moreover, the tightness of control was maintained also at high vector doses (33,60). Dox-dosing experiments demonstrated that the extent of Epo gene expression, and of the consequent Hct increase, could be finely modulated over a 1-year period by varying oral Dox dosage (33). The reliability of the $rtTA2^{S}$-M2/tTS system was confirmed by the tight control of SEAP expression observed in Rhesus monkeys upon intramuscular electroinjection of plasmid DNA (33). Of interest for potential application in humans, the system was fully induced in mice and nonhuman primates by Dox-dosing regimens comparable to those used in the clinical practice (33).

E. Inducer Drugs

A major advantage of tetracyclines is the broad knowledge we have about their pharmacological and chemical properties, as several members of this class of compounds have been used in human and animal medicine for decades. As indicated above, doxycycline efficiently stimulates both the Tet-OFF and the Tet-ON system, and was therefore used as the effector of choice in animal models.

With its efficient oral absorption (93%), prolonged half-life (14–22 h), and excellent tissue penetration, Dox would be the ideal agent also for controlling the Tet-system in a human gene therapy setting (64). Experiments in animals (see above) and theoretical considerations strongly indicate that the latest versions of the ON systems, which are fully activated at Dox concentrations of about 200 ng/ml, will be efficiently stimulated in vivo by the Dox therapeutic regimen commonly used for antibiotic therapy. In fact, administration of 200 mg of Dox in humans produces peak serum concentration of 3 μg/ml after 2 h, with a plateau-shaped concentration curve that remains over 1 μg/ml at 24 h. Also, lower dosages should be effective: for instance, following administration of 20 mg Dox, twice a day, the mean peak concentration in plasma is about 800 ng/ml and the average steady-state concentration is about 400 ng/ml (64) and references therein]. Because of its capacity to penetrate the blood-brain barrier, Dox has the potential to modulate gene expression in the central nervous system (CNS), as extensively demonstrated in animals. Finally, the principal excretory organ for Dox is intestine, thus rendering this drug useful also in cases of preexisting renal disfunction (64).

Dox is usually well tolerated: the most serious side effect is photosensitivity (characterized by overreaction to sunburn). It is dose-dependent and may occur in 1–3% of the patients after prolonged (several months) therapy. Since Dox accumulates in the bones, it may cause yellow-brown coloration of the teeth. In conclusion, Dox has an excellent safety profile for short-term (i.e., a month or less) applications: less is known on the potential adverse effects following more prolonged administration.

Finally, the major concern on the use of an antibiotic-driven regulatory switch is the possibility of raising resistance to the antibiotic itself (64). Actually, tetracyclines are less used than in the past, but may represent the treatment of choice for serious diseases, like anthrax, Lyme disease and Rickettsial disease. Therefore, nonantibiotic Dox analogs capable of activating the Tetsystem would be desirable in a clinical context.

F. Perspectives

During the past years the tet system has been significantly improved in terms of basal activity, inducibility, and sensitivity to the inducer drug. The latest versions of the Tet-ON activators and the tightness guaranteed by the tTS suppressor have significantly enhanced the potential of this system for gene therapy applications.

An attractive feature of the system is its flexibility, which allows generation of a set of TetR protein derivatives with different phenotypes, but which still maintains the main characteristic of the wt protein: ligand-regulable binding to a specific DNA sequence. An example was the construction of tTA and rtTA homodimers that recognize different promoters and respond differentially to the various tetracyclines (65,66). We can thus expect novel versions of the Tet-Off and Tet-On system to be available in the future.

The Tet system uses prokaryotic elements and might therefore prove immunogenic in humans. It has been demonstrated that activators could be partially humanized by substituting the VP16 AD with ADs derived from human proteins (such as the p65 activation domain from human NF-kB and the activation domain from E2F4) without loss of efficacy (67,68). Nonetheless, the core of the activators is of bacterial origin and its immunogenic potential may limit its application to the short-term range in humans.

Until now, no immune response in mice against the tet system delivered with viral and nonviral vectors in a great variety of tissues has been reported. On the contrary, recent studies suggest that intramuscular injections of plasmids or viral vectors expressing Dox-dependent activators may elicit a cellular and humoral response against Tet-ON activators in nonhuman primates (58,69). Of course, this is an issue of primary importance and must be carefully and extensively studied in further works. Among the possible strategies that could be applied to reduce the potential immunogenicity of the activators are: immunosuppression of the host at the time of vector delivery, use of vectors with low intrinsic immunogenicity, such as AAV, and using tissue-specific promoters to restrict expression of the exogenous genes to nonprofessional antigen-presenting cells. Testing these and other strategies in nonhuman primates will probably be the major challenge in the near future.

III. NOVEL INDUCIBLE EUKARYOTIC GENE EXPRESSION SYSTEMS BASED ON PROKARYOTIC ELEMENTS

In recent years significant efforts have been directed to developing additional transcription systems which, along the same basic principle exploited by Bujard and colleagues (26), utilize well-known, ligand-induced, transcriptional activators taken from the bacterial world and convert them into eukaryotic transcription factors via simple manipulations such as the addition of ADs. The advantage of these systems is that they rely on DNA binding proteins that: a) do not interfere with eukaryotic transcriptional networks, b) recognize DNA binding sites that are not bound by mammalian transcription factors, and c) are capable of binding with high affinity distinct classes of small molecular weight ligands, some of them already used in the clinic. Although these new systems have not yet reached the same level of preclinical characterization of the Tet system, they show highly interesting properties for future translation to clinical applications.

A. A Switch Based on the Quorum-sensing Transcription Factor TraR

Bacteria are capable of ''sensing'' fluctuations in their population density, and respond by changing the pattern of gene expression. This response, called Quorum Sensing, is based on a simple mechanism consisting of a signal molecule, an acylated homoserine lactone (AHL) that accumulates in the immediate external environment, and a cognate transcription factor that it activates (70). Over 50 species of gram-negative bacteria produce AHLs that differ in the acyl side chain: an attractive feature of this two-component regulatory system is the existence of several variants in nature. The bacterial quorum sensing system represents a natural combinatorial library that could be exploited to generate reagents for the development of artificial eukaryotic gene regulation systems. To this end, it is necessary to reengineer the prokaryotic basic module, i.e., the signal and its cognate ''receptor,'' so it may function in eukaryotic cells.

TraR belongs to the LuxR family of transcriptional activators (70,71,72). Binding the small, diffusible *Agrobacterium* quorum-sensing signal 3-oxo-C8-HSL (Fig. 6) results in TraR activation and subsequent interaction with promoters containing one or more copies of an 18-bp inverted repeat called *tra* box (73–75). The 3-dimensional structure of TraR has recently been solved. The protein consists of two separate domains; an N-terminal domain with ligand-binding and dimerizing properties, and a C-terminal domain that binds DNA (76,77). This modular structure makes the LuxR family of transcriptional activators a promising candidate for genetic manipulation.

A hybrid transcription factor was generated by fusing the activation domains VP16-F3 or p65 N-terminally to the cDNA fragment corresponding to the TraR wt protein (34,78). Both activation domains were separated from TraR by a short amino acid linker (79). In addition, a nuclear localization signal was added to the linker region of p65 in order to ensure nuclear localization of the chimeric protein. As in the case of authentic TraR, the chimeric transcription factors bind to DNA containing a *tra* box only in the presence of a cognate ligand, such as 3-oxo-C8-HSL (79). Importantly, 3-oxo-C8-HSL must be present during the protein synthesis reaction, in accordance with previous findings that the ligand is necessary for proper folding of TraR (80,81). In fact, the 3-dimensional structure shows that the ligand-binding site is deeply embedded in the protein core. This requirement brings the advantage that the system is totally silent in the absence of the ligand, but is

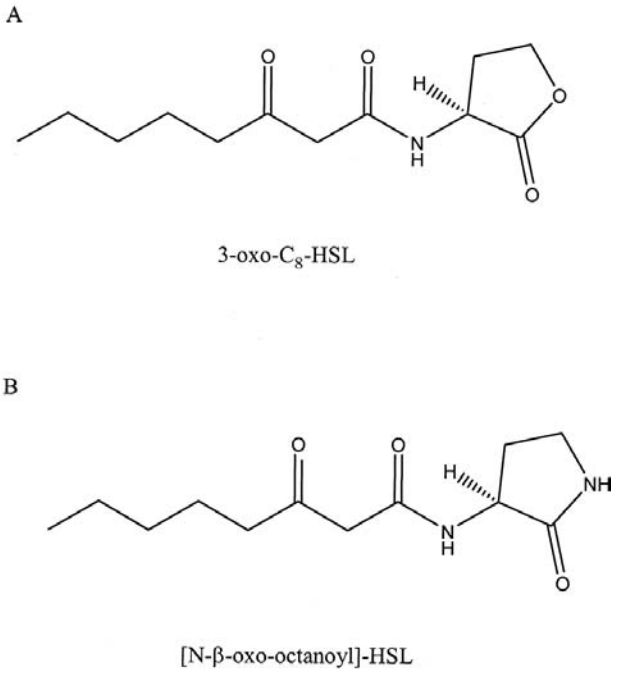


Figure 6 Chemical structure of acylated homoserine lactone molecules, inducers of the TraR-based system. (A) *Agrobacterium* quorum sensing *N*-(3-oxo-octanoyl)-homoserine lactone (3-oxo-C_8-HSL); (B) Synthetic [N-β-oxo-octanoyl]-homoserine lactam.

limited by there always being a substantial time lag between cell exposure to AHL and gene activation.

It was observed that the affinity of 3-oxo-C8-HSL for the ligand-binding domain of TraR does not significantly change in the context of the chimeric protein. However, the apparent affinity of 3-oxo-C8-HSL for TraR in eukaryotic systems is lower than in bacterial strains (μM vs. nM, respectively) (79). The reason for this is not presently understood, and future work will be needed to address this issue. P65NtraR and F3-TraR induced transcription of gene clones downstream of TraR-responsive promoters in eukaryotic cells (Fig. 7). The highest inducibility was obtained with p65NtraR acting on a target promoter consisting of seven *tra* boxes. The leakiness was low, and adding 3-oxo-C8-HSL to the cell culture medium led to strong induction of gene expression, comparable to the activity of the constitutive CMV enhancer/promoter. Robust induction of SEAP expression in the presence of p65NtraR and 3-oxo-C8-HSL was shown in different human cell lines, and TraR induction was dose-dependent for 3-oxo-C8-HSL, reaching up to 1000-fold induction (79).

A lactone moiety, which undergoes spontaneous lactonolysis upon prolonged exposure to aqueous conditions (82), is invariably present in all the quorum sensing signal molecules of the LuxR family of transcriptional regulators (70). The limited chemical or enzymatic stability of lactones thus poses an obstacle to future development of this system. In the attempt to design a ligand more suitable for experiments in eukaryotes, Nedderman et al. (79) synthesized [N-β-oxo-octanoyl]-homoserine lactam (Fig. 6). Notably, the [N-β-oxo-octanoyl]-homoserine lactam was stable under experimental conditions and nontoxic up to a concentration of 500 μM. The affinity of p65NtraR for the lactam was about 10-fold lower than that for 3-oxo-C8-HSL (EC_{50} 70 μM vs. 6 μM); nonetheless, because of the nontoxic nature of the compound, strong induction was achieved by using higher concentration (hundreds μM) of the compounds (79). Thus, in spite of the striking conservation of the lactone ring in natural quorum sensing signal molecules, it is possible to develop compounds that combine increased stability with biological activity.

In conclusion, although the characterization of this new system is still preliminary, its low basal transcriptional activity and the robust dose-dependent induction offer significant advantages. Its general properties suggest that it is perhaps possible to engineer other LuxR proteins that respond to different AHLs and thus increase the repertoire of genes whose expression could be simultaneously regulated, provided that the cognate AHL for one LuxR protein does not activate the others.

B. Streptogramin- and Macrolide-responsive Systems

In recent years, M. Fussenegger and colleagues have developed two regulatory systems which, similar to the Tet system, are based on naturally evolved mechanisms of antibiotic resistance.

1. Streptogramin-responsive System

A gene regulatory system was developed based on a Streptomyces antibiotic resistance operon (83). To tolerate their own antibiotic products, Streptomyces have developed various resistance mechanisms. A pristinamycin resistance operon was identified and cloned from *S. pristinaespiralis*. *S. pristinaespiralis* produces pristinamycin, a composite streptogramin antibiotic consisting of a pair of structurally unrelated molecules: pristinamycin I (PI, also called Streptogramin B), a cyclic hexadepsipeptide, and pristinamycin II (PII; also called Streptogramin A), a polyunsaturated macrolactone (84). *S. pristinaespiralis* genome codes for an efflux-type pristinamycin-resistance determinant (*ptr*), whose expression is induced by pristinamycin itself. A repressor protein, called Pip (pristinamycin induced protein), binds to the *ptr* promoter (Ptr) and represses transcription of the resistance gene. In the presence of pristinamycin, the antibiotic binds to Pip, and this results in the release of Pip from Ptr and induction of gene expression (85). Similar to the Tet system, the pristinamycin repressible Pip-Ptr interaction was adapted for use in mammalian cells. Two configurations were developed: PipOFF and PipON (Fig. 8).

In the PipOFF system, a pristinamycin inducible transactivator (PIT) was obtained by fusing Pip to the VP16 activation domain, and a PIT responsive promoter, called Ppir (pristinamycin repressible promoter) was constructed by combining

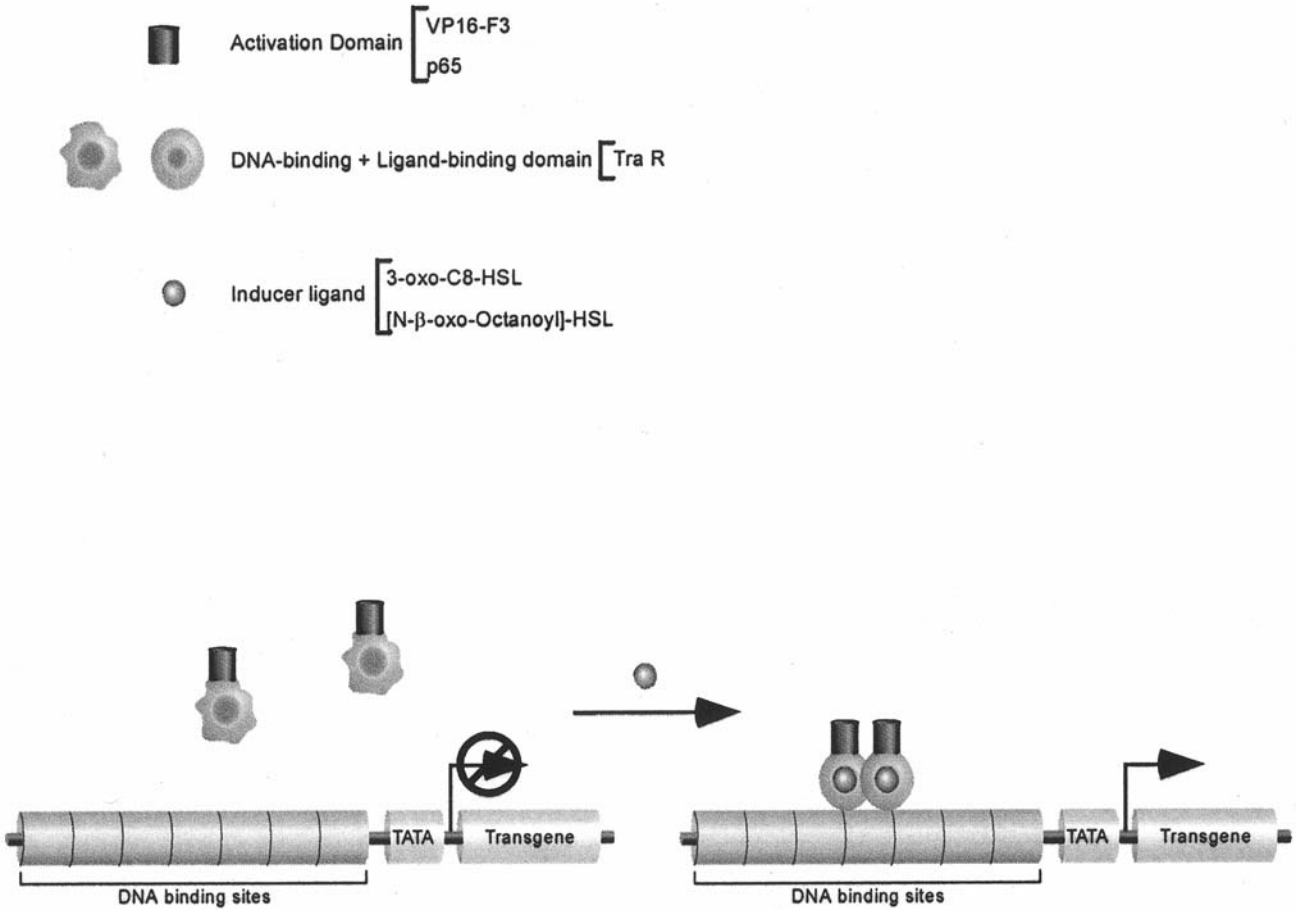


Figure 7 TraR-based regulatory system. The activator consists of TraR fused to an activation domain. In the absence of the ligand, the protein is unfolded: drug administration promotes appropriate folding of the chimera, which acquires the capability of binding and activating a target promoter containing multimeric TraR binding sites. See text for additional details.

three Pip operator sequences with a minimal promoter from Drosophila heat-shock protein 70 gene ($P_{hsp70min}$). In the absence of the ligand, PIT binds and activates Ppir, whereas the system is silenced in the presence of the antibiotic, which disrupts PIT-Pir interaction (83).

The PipON system is based on the use of Pip coupled with a pristinamycin-inducible promoter (PpirON, also called PSV40-PIR3), which consists of multiple Pip operator sequences cloned downstream of the SV40 strong constitutive promoter. In the absence of the antibiotic, Pip binds to PpirON and blocks the SV40 driven expression: in the presence of the antibiotic, Pip is released and gene expression induced. In the PipON configuration, fusing Pip with the KRAB repressing domain further enhanced its repressing capability: in this configuration, the system displays a 10-fold lower basal activity but also a reduced maximal activity (83).

Both systems have so far been tested only *ex vivo* in mammalian cells, by transient transfection or retroviral infection. They enabled hundreds-fold induction of reporter gene expression and displayed a relatively low basal activity. Interestingly, the PipON and PipOFF systems are only responsive to PI (Fig. 9), while PII had no effect. This finding is of interest when translated into a human therapeutic context: in fact, the use of PI alone as a stimulatory molecule is unlikely to elicit broad antibiotic resistance against composite streptogramin (PI + PII) (85).

2. Macrolide-responsive System

More recently, Fussenegger and colleagues developed a system regulated by macrolide antibiotics, such as erythromycin (EM), whose structure is shown in Fig. 10. Also in this case, the key elements derive from an antibiotic resistance operon. The *Mph(A)* gene codes for a 2′-phosphotransferase that inactivates 14-membered macrolides (85). Its expression in basal conditions is repressed by a prokaryotic repressor, MphR(A), which binds to a 35-bp operator sequence (ETR) overlapping

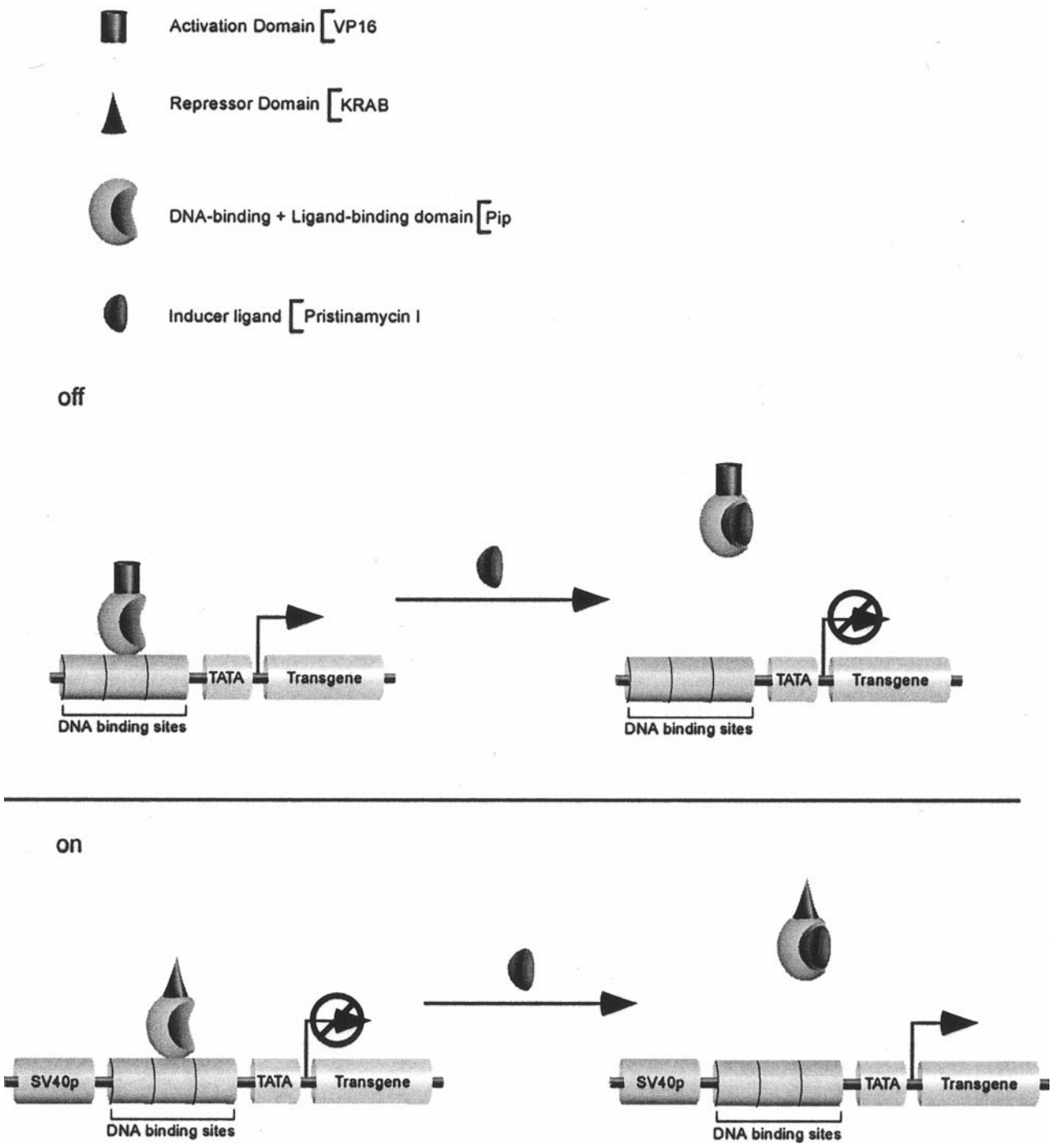


Figure 8 Streptogramin-responsive system. PipOFF: the chimeric activator (called PIT) consists of Pip fused to an activation domain. In the absence of pristinamycin, it binds and activates the target promoter consisting of three Pip binding sites (operator sequences) upstream of a minimal promoter. In the presence of the ligand, PIT is released from DNA and transcription is terminated. PipON: the chimeric repressor consists of Pip fused to the KRAB repressor domain and the target promoter consists of Pip operator sequences cloned downstream of the SV40 promoter. In the absence of the drug, the Pip-KRAB fusion binds to its target sequences, thus silencing the SV40 promoter. Drug administration results in release of the Pip-KRAB fusion from DNA and consequent derepression of the SV40 promoter. The macrolide-responsive system operates according to the same principles. See text for additional details.

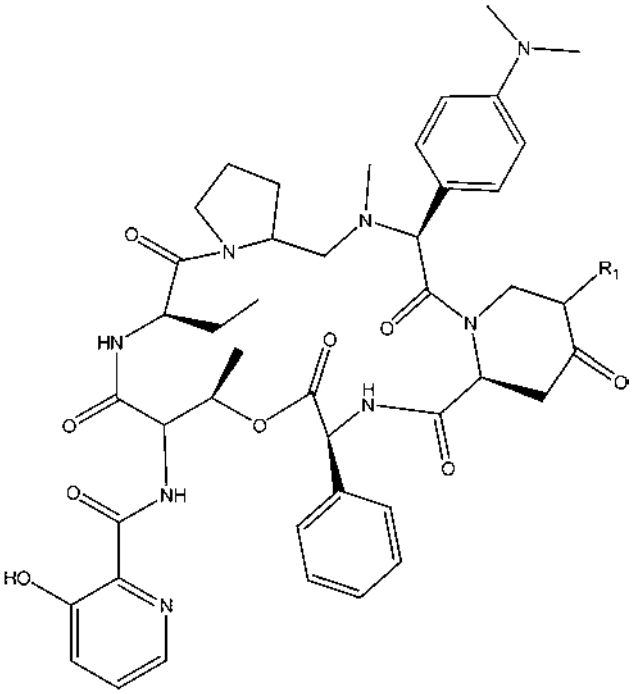


Figure 9 Chemical structure of pristinamycin I (PI), inducer of the streptogramin-responsive system. See text for additional details.

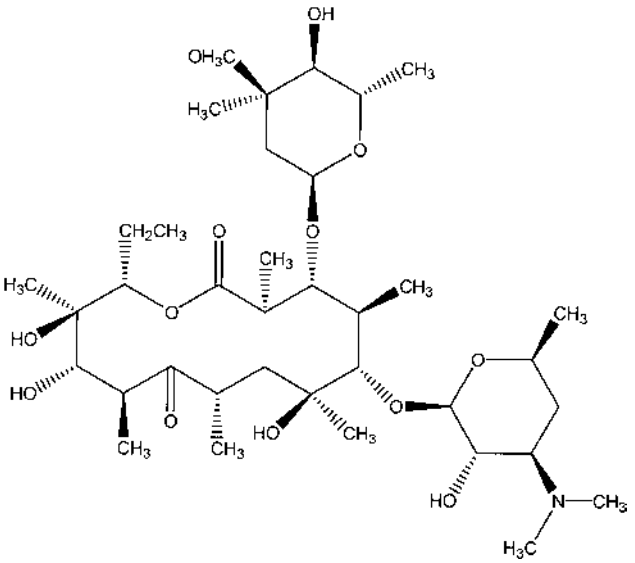


Figure 10 Chemical structure of erythromycin, inducer of the macrolide-responsive system. See text for additional details.

the *Mph*(*A*) promoter. Upon binding to EM, MphR(A) dissociates from its target DNA sequence, thus allowing transcription of *Mph*(*A*) gene (85).

Very similarly to the streptogramin system, Erythromycin ON (E_{ON}) and OFF (E_{OFF}) systems were generated (86). The E_{OFF} system is based on an erythromycin dependent transactivator ET1, a fusion between MphR(A) and VP16, which binds to an erythromycin responsive promoter (P_{ETR}) consisting of the *Mph*(*A*) operator fused to $P_{hsp70min}$ minimal promoter. The E_{ON} switch relies on MphR(A)-KRAB fusion binding to an artificial ETR octamer cloned downstream of the SV40 promoter. As expected from the ligand-binding and DNA-binding properties of MphR(A), and similar to the streptogramin systems, the E_{OFF} and E_{ON} systems are activated by drug withdrawal and administration, respectively. Also in this case, MphR(A)-KRAB fusion proteins increased the stringency of the E_{OFF} system. Hundreds-fold induction and low basal activity were reported in transient and stable transformants. In addition, both the on and off systems proved capable of regulating Epo expression in an EM dose-dependent manner from intraperitoneally injected stable transformants carrying the regulatory system and the regulated Epo gene (86).

Both the streptogramin- and the macrolide-responsive systems are still in their infancy and we can predict that, as happened with other systems, they will be progressively improved in terms of inducibility, potency, and drug-responsiveness. In line with this prediction is the recent report that the performance of both systems can be dramatically improved by introducing minor modifications to the molecular architecture of their target promoters (87).

An attractive feature of both systems is that they are activated by clinically licensed compound, which may facilitate their use, especially for short-term applications. As for the Tet system, the ON configurations have greater potential for gene therapy applications, but additional work in preclinical models will be required to more precisely evaluate the positive and, possibly, negative features of both systems.

IV. DIMERIZER-REGULATED SYSTEM

A. General Principles

The initial concept of using chemical dimerizers of protein-protein interaction in order to activate biological processes is due to the pioneering work of S. Schreiber and G. Crabtree (88,89). However, its further evolution and adaptations to a variety of applications, including transcriptional activation, is due mainly to the activity of the ARIAD Gene Therapeutics Inc. group. Several recent reviews have nicely summarized the work in this area (90–92).

The foundation of this system is the use of chemical dimerizers, small chemical entities with two distinct binding surfaces for identical or different polypeptides (89,92). The prototype molecule utilized for this purpose, FK1012, is a homodimer of the immunosuppressant drug FK506. It bears two identical, high-affinity, protein-binding surfaces for FKBP12, a 12-kDa cytoplasmic protein (88,89). FK1012 as

a protein homodimerizer is utilized mainly for ligand-induced receptor dimerization and activation of intracellular signaling, thanks to the use of chimeric receptors that contain the ligand-binding domain of FKBP12. These synthetic receptors have been engineered to undergo dimerization in the presence of FKBP binders but not of endogenous ligands (88,89,93).

The modular nature of eukaryotic transcription factors enabled the construction of a dimerizer-induced regulatory system (94). DNA Binding Domains (DBDs) and Activation Domains (ADs) can be expressed independently of each other. Each domain maintains its proper folding: a DBD bound to its target promoter is unable to activate transcription unless the AD is placed in close proximity. This simple concept has been extensively exploited in the setting of two-hybrid assays in yeast (95). By fusing a DBD and an AD with a drug-binding domain, assembling an active transcription factor thus depends on adding of a dimerizer-ligand capable of bridging the DBD and AD by simultaneously interacting with the drug-binding domains of the fusion proteins. In this context, however, fusion of the DBD and the AD with the same ligand-binding domain and the use of homodimerizing drugs are not appropriate, as it would also lead to the generation of inactive DBD-DBD and AD-AD homodimers. To prevent homodimerization, this system has been evolved to incorporate chemical heterodimerizers. Since no chemical entity is known with sufficient affinity and selectivity to bring together a DBD and an AD, attention has been directed to utilizing the natural targets of known chemical heterodimerizers. One such molecule is another immunosuppressant drug, rapamycin, a natural product macrolide (Fig. 11).

Rapamycin binds the immunophilin FKBP12 with a nanomolar affinity (96), and this complex interacts with FRAP (FKBP rapamycin-associated protein) (97) also called mTOR (mammalian target of rapamycin), a PI3-kinase homolog involved in control of cell growth and division (98). FKBP and an 89-aa fragment of FRAP, called FRB, sufficient to bind the FKBP-rapamycin complex (99), can be linked to a DBD and to an AD, respectively. These two components, namely DBD-FKBP and FRB-AD, associate noncovalently in the presence of rapamycin. A gene cloned downstream of a promoter containing sites recognized by the DBD-FKBP fusion is thus only transcribed in the presence of the dimerizer drug, according to the scheme shown in Fig. 12.

B. Transcription Factors

The dimerizer-regulated system was constructed by using protein domains of human origin in order to minimize its potential immunogenicity. DBD-FKBP and FRB-AD were thus generated by using human protein domains. The two heterodimerizing components FKBP and FRB are human proteins. The selected DBD, ZFHD1, is a composite DNA-binding domain with novel DNA recognition specificity (100). It is a 122 polypeptide constituted by a 58 aa zinc-finger domain from the natural human Zif268 zinc-finger protein linked via two glycine residues to the 62 aa POU domain of the human transcription factor Oct-1. The AD is derived from the carboxy-terminal region of the human transcription factor NF-kB p65 (78). The two transcription components have been called ZFD1-FKBP (the version that carries three tandem copies of FKBP, named ZF3, was initially selected because of its excellent drug-dependent activity in transfected cells) and FRB-p65, respectively (also called S1) (101). They can be expressed either from separate transcription units or from a single bicistronic transcript by means of the ECMV (encephalomyocarditis virus) internal ribosome entry site (IRES).

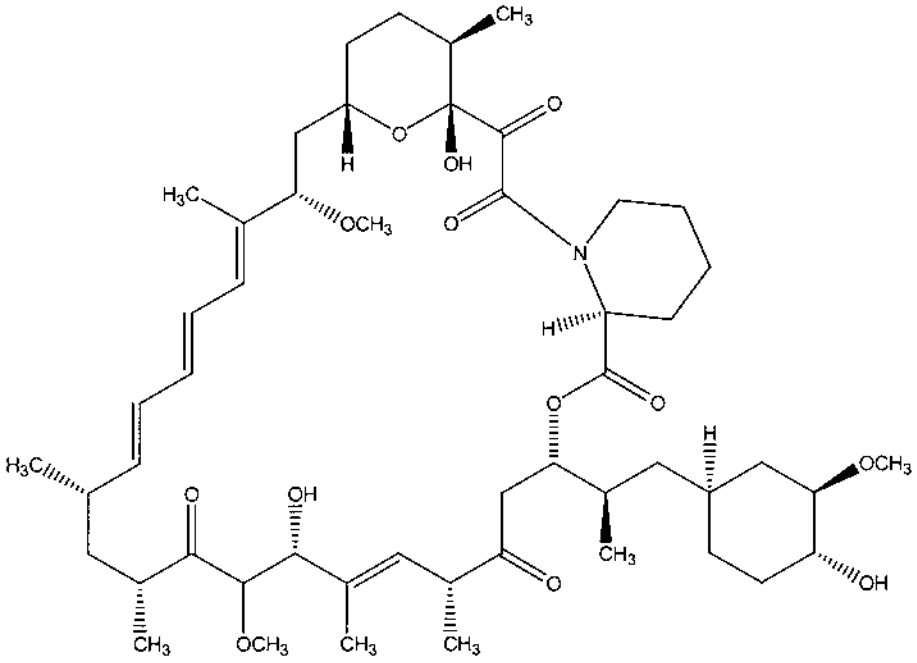
Further modifications/improvements of these transcription factors have been obtained, through the generation of more potent FRB-carrying components. A widely accepted concept is that enhancing the efficiency with which activators are delivered to a promoter strongly potentiates transcription efficiency (102). Linking two ADs, namely those of human p65 and of human heat shock factor 1, to FRB, generated a more potent factor (103). Use of this more potent factor may be appropriate when higher sensitivity is required, for example when low gene copies/cell are transduced by retroviral vectors (103). A second elegant example was the generation of FRB-p65 fusions that carry a portion of the *E. coli* lactose repressor between the two domains that form tetrameric bundles and therefore multimers of the AD (104). These improved activators induce higher maximum expression levels of the regulated reporter genes and are more responsive to rapamycin. However, while the first solution is entirely based on human proteins, the second suffers from the potential immunogenicity of its *E. coli*-derived component.

More recently, a system similar to that induced by rapamycin has been described that responds to nonimmunosuppressive analogs of FK506. FK506 heterodimerizes FKBP and calcineurin, a protein phosphatase that mediates the mitogenic stimuli from the T cell receptor (105). The FK506-FKBP binding interface of calcineurin is composed of aminoacid residues that belong to its component polypeptides, called can and CnB. Based on the X-ray structure of the FKBP-FK506-can-CnB complex, protein design was used to generate a synthetic calcineurin A-B fusion, called mCAB, which binds FKBP-FK506. A yeast three-hybrid system was then used to identify mCAB mutants that interact with FK506 analogs unable to bind calcineurin and therefore not immunosuppressive. A regulatory switch based on DBD-FKBP and mCAB-AD components was significantly activated by these FK506 analogs in transiently transfected cells (106). The potential of this system for gene therapy applications remains to be verified (see below).

C. Dimerizer-responsive Promoters

As with other ligand-dependent regulatory systems, dimerizer-responsive promoters are essentially composed of a downstream element, the minimal promoter, and an upstream element with a variable number of binding site(s) for the inducible transcription factor. The downstream elements used are

A



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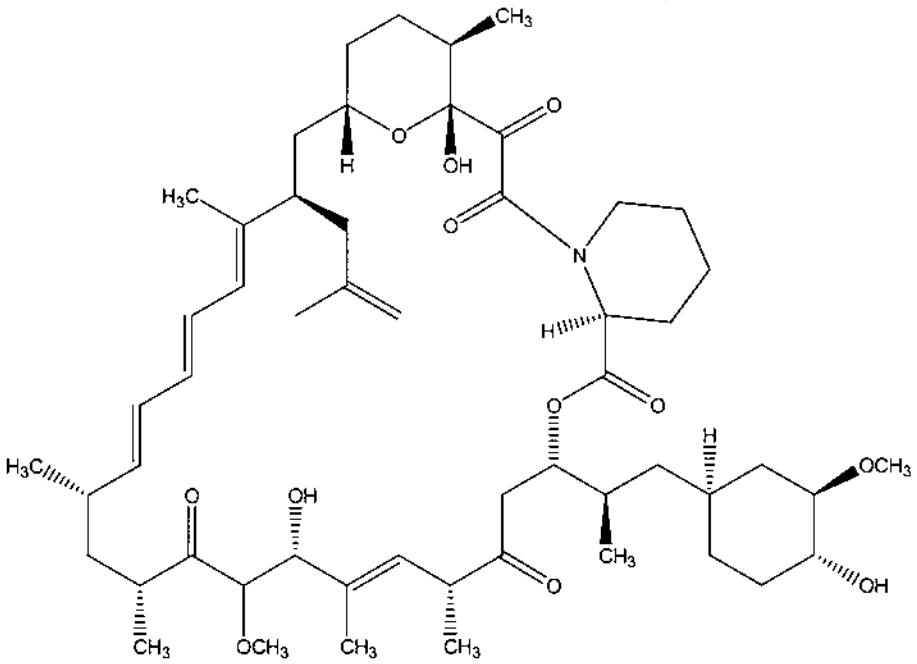

Figure 11 Chemical structure of dimerizer-dependent system inducers. (A) Rapamycin, the more commonly used inducer of the system; (B) Rap*, nonimmunosuppressive rapamycin-derivative.

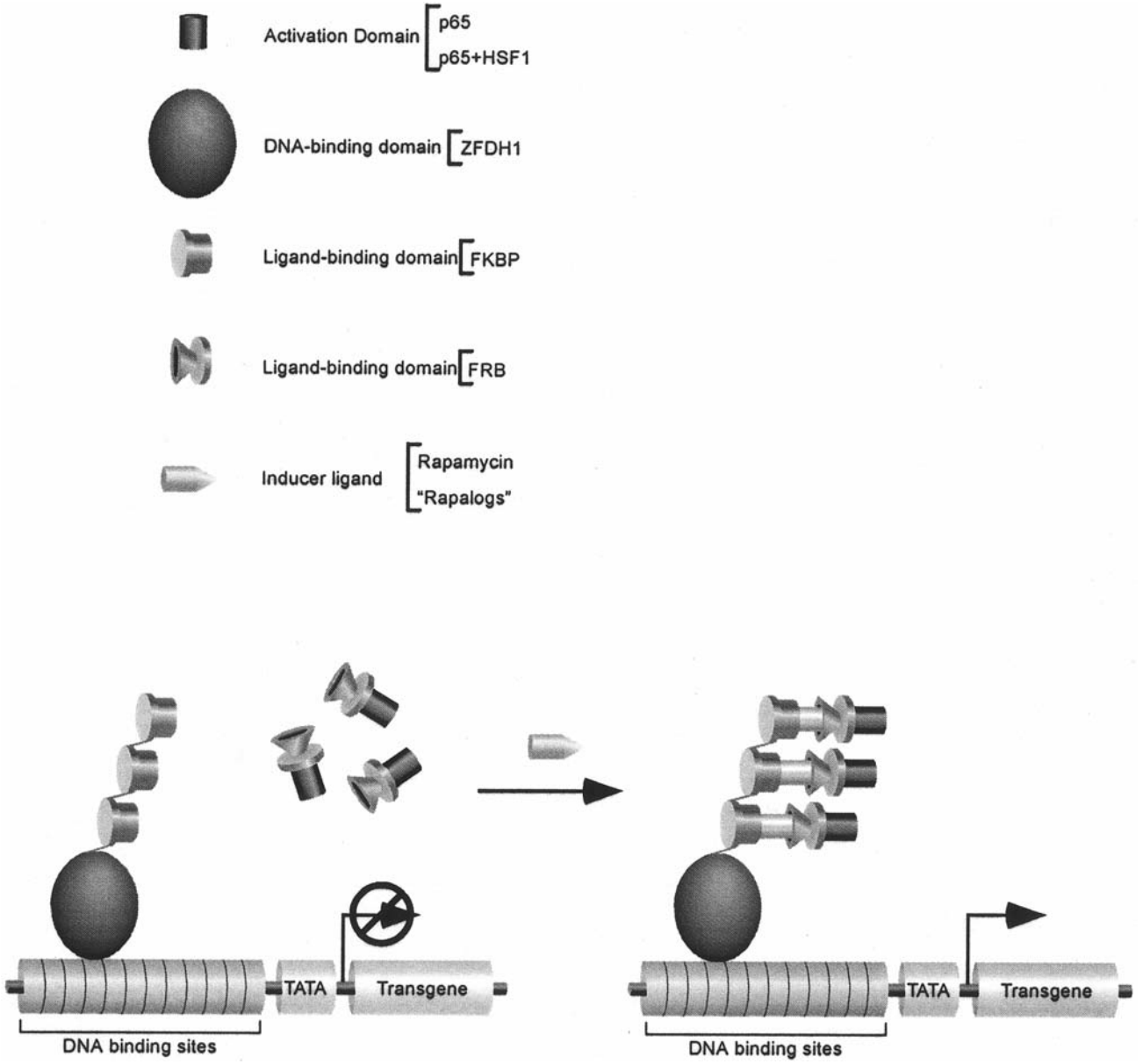

Figure 12 Dimerizer-dependent system. One component is a DNA binding domain consisting of a composite zinc-finger homeodomain (ZFHD1) fused to three FKBP repeats. The second component is an activation domain fused C-terminally to the FRB domain. The two fusion proteins can be joined by heterodimerizer drugs, thus generating a composite transactivator that activates a target promoter consisting of 12 ZFHD1-binding sites.

commonly the minimal cytomegalovirus immediate early promoter (101), or the minimal IL-2 promoter (107).

The upstream element is the binding site for ZFDH1. As described above, ZFDH1 is a synthetic DBD generated by protein design. Its consensus DNA-binding site (DBS), 5′-TAATTANGGGNG-3′, has been derived by selection from a random pool of oligonucleotides (100). The particular juxtaposition of its two components, the two zinc fingers from Zif268 and the POU domain of Oct-1, allows ZFDH1 to selectively bind the sequence 5′-TAATGATGGGCG-3′ with a dissociation constant of 8.4×10^{-10} M, an affinity at least 30-fold higher than that of either Zif268 or Oct-1 for the same sequence. Hence, the ZFDH1 DBS is not recognized by endogenous transcription factors: multimers of this DBS linked

to a minimal promoter and to a reporter gene, when transfected in cells, do not give rise to detectable reporter gene expression (101). The most commonly used promoter configuration contains 12 tandem copies of the ZFDH1 DBS (Z12).

D. Dimerizer-regulated System for Gene Therapy Application

In general, dimerizer-based systems display a very low basal activity in the absence of the inducer drug. The main reason for this is the physical separation of the DBD from the AD in two distinct fusion proteins that cannot interact in the absence of ligand. This tight regulation was constantly observed in cells after transient or stable transfection, and also when stably transfected cells were implanted in vivo at various dosages (101,108). The tightness of the system was demonstrated by efficient generation of stable cell lines carrying an inducible highly toxic gene (103).

Since basal transcription ranges from low to undetectable, induction ratios in the presence of rapamycin are very high (up to 1000-fold) in cultured cells. The dose-response curve with the standard system, i.e., ZF3/S1, reaches maximal induction in stably transfected cells at approx. 50 nM rapamycin with an EC_{50} of 6–8 nM. Maximal gene expression levels are comparable to those obtained with constitutive strong promoters like the CMV promoter/enhancer (107). However, in a comparative study with the rtTA2-M2-based tet-on system, the rapamycin system displayed lower leakiness but also lower maximum induced activity after transient transfection (109).

The dimerizer-regulated switch enabled tight control of gene expression also when delivered in various animal models of gene therapy using viral vectors (mainly AAV), moving from small animals to nonhuman primates (Table 2). When delivered by AAV vectors in mouse muscle, the system enabled long-term and strictly rapamycin-dependent control of GH and Epo (107,110). Importantly, varying dosage of the inducer drug modulated gene expression (107,110). Inducible expression was also demonstrated in mouse eye and liver following AAV and adenoviral gene transfer (111,112).

Similar studies were also performed in nonhuman primates: of particular relevance was the demonstration that a regulated macaque Epo gene delivered with AAV vectors in the muscle of rhesus macaques could be repeatedly induced upon systemic drug delivery (110). Inducibility of transgene expression was not maintained over a prolonged period of time in all animals: out of six primates tested in total, three underwent expression shut-off. However, it has been recently reported in an extended trial involving eight animals injected with an improved system that all of them show persistent expression (92). This is a preliminary indication that the shut-off observed in the first study may not be due to immunological response against the activator but, possibly, to decreased expression of the two transactivators below a therapeutic threshold.

Further confirmation of this system reliability comes from the inducibility of Epo expression in the anterior chamber of macaque eye after subretinal injection of AAV vectors (112).

A recent report described use of the rapamycin-dependent system to control replication of adenoviral vectors in cancer cells. Conditionally replicating adenoviral vectors have been recently introduced in clinical trials with promising results (113). These vectors are designed to undergo replication and spread only to cancer cells. For this purpose, intratumoral, loco-regional, or also systemic delivery are being investigated. However, safety concerns have raised the interest in pharmacological regulation of replicating adenoviral vectors. A recent study has shown that it is possible to engineer a conditionally replicating adeno in which the E1a gene is put under the control of a minimal promoter with 12 ZFDH1 sites (114). Direct coinjection in tumor cells of this virus with a first generation adeno expressing the two hybrid transcriptional activators ZH3 and S1 leads to rapamycin-inducible adenovirus replication (114). Although therapeutic efficacy in a xenograft model has not yet been convincingly shown, and the current system requires two complementary adenoviruses, a refined version in which all these elements are contained in a single vectors can be envisaged as a new weapon in the fight against cancer.

E. Inducer Drugs

Rapamycin is the principal ligand used to stimulate the dimerizer-based regulatory system, which indeed is commonly known as the ''rapamycin system.'' Its pharmacological and pharmacodynamic properties are well known (115). It is orally bioavailable (see below) and has a half-life of 4.5 h. A fairly extensive characterization of the kinetics of transgene induction-deinduction in vivo was performed in mice after IM implantation of stable clones expressing the hGH under the con-

Table 2 Dimerizer-dependent System: In Vivo Studies in Animal Models of Gene Therapy

Transactivator	Transgene[a]	Vector	Tissue	Species	Refs.
ZF3 + FRAP-S1	GH	Ad, AAV	muscle	mouse	107
ZF3 + FRAP-S1	Epo	AAV	muscle	mouse, monkey	110
ZF3 + FRAP-S1	Epo	AAV	retina	rat, monkey	111
ZF3 + FRAP-S1	Ins	Ad	liver	mouse	112
ZF3 + FRAP-S1	E1a, E1b	Ad	tumor xenograft	mouse	114

[a] GH = Growth Hormone; Epo = erythropoietin; E1a, E1b = adenovirus early genes; Ins = insulin.

trol of the dimerizer system. After a single IV or oral administration, peak transgene levels were reached after 24 h, maintained for the following 48 h, and then started to decline with a half-life of one day (108). Considering that the half-life of GH is only minutes and that of rapamycin is 4.5 h, this extended kinetic of expression is probably due to the rapamycin-mediated complex between the chimeric DBD and AD being highly stable for several hours in the cell nuclei. As a consequence, transcription shut-off in response to drug withdrawal is a slow process, which depends on degradation rate of the two transcription factors via the proteasome pathway. The intrinsic inertia of the dimerizer system may thus render daily drug administration superfluous to keeping the system in the on state. On the other hand, this also precludes prompt silencing of the system by drug withdrawal if and when required.

Rapamycin is only partially orally bioavailable (115). Maximal activation is observed at 10-fold higher doses after oral delivery than by parenteral administration and, while the ED_{50} derived from intravenous administration is slightly above 1 mg/kg, that derived from oral dosing is 9–10 mg/kg. This explains why in the majority of published studies the ligand was administered by intraperitoneal injection (107,108,110–113). Indeed, at the common doses of 1–2 mg/kg used in small and large animal models, transgene expression is already half-maximally activated after parenteral injection of rapamycin, while only marginally induced when the drug is given orally (108). Highly immunosuppressive oral doses would thus be required to reach therapeutic levels of the target transgene in humans, thus making this version of the system impractical for clinical applications. These high concentrations of the ligand might also interfere with the physiological functions of FKBP, such as calcium channel modulation and cardiac development (116,117). The potential solution to these issues is to use rapamycin analogs that maintain the capacity to heterodimerize DBD-FKBP and FRB-AD but have lost their immunosuppressive properties.

Immunosuppression by rapamycin is due to the inhibition of FRAP enzymatic activity (118). The 3-dimensional structure of the ternary complex of FKBP, rapamycin, and FRAP is known, and has helped redesign the binding interface of rapamycin with FRAP (119). Bulky substituents were introduced in the C16 position of rapamycin, which abolished binding to wt FRAP and, as a consequence, its immunosuppressant properties. Likewise, compensatory mutations were obtained by genetic selection in yeast of a FRAP mutant library. These restored binding of a C16 substituted nonimmunosuppressant rapalog called Rap*, carrying a methallyl substituent (Fig. 11) (120). A triple substitution mutant, T2098L/W2101F/K2095P, called FRB*, was shown to efficiently and specifically bind the FKBP-Rap* complex. When the FRB*-p65 fusion was used in transfected cells as a replacement of the original FRB-p65 component, it induced target gene expression in the presence of Rap* at an EC_{50} below 10 nM.

Other rapalogs substituted in the C7 position have been obtained (121). Although their structure has not yet been disclosed, they are assumed to act in combination with a single aminoacid-substituted FRB T2098L. This preferred FRB variant (called $FRAP_L$) and some of its corresponding rapalogs AP22565 and AP21967 have been shown to efficiently induce gene expression in cell lines transduced with a retroviral vector or in vivo in tumors injected with conditionally replicating adenoviral vectors (103,114). Based on these results, it would seem the solution to immunosuppression at hand. However, it has to be kept in mind that changes to the structure of rapamycin may modify its pharmacology and drug metabolism. Until now, no transcription-specific rapalog has been reported with a defined oral bioavailability and acceptable pharmacokinetics.

F. Perspectives

This class of transcriptional activators and their related ligands are of relatively new conception but have quickly acquired importance as highly promising for in vivo gene therapy applications and future clinical development. The system embodies most of the properties required, i.e., low basal activity in the absence of the ligand, high dynamic range, and full reversibility of the transcription switch upon ligand withdrawal. Its level of characterization in in vivo preclinical models is fairly advanced, and the intrinsic flexibility and modularity of its components offer opportunities for future evolution.

In particular, zinc-finger technology has made such significant progress over the past years that it is now possible to engineer zinc-finger domains targeted to any desired DNA sequence (122). These novel polypeptides can be used to activate or block gene expression when targeted to open chromatin domains of resident genes. By using appropriately designed zinc fingers, it may thus be possible to generate dimerizer-regulatory systems capable of specifically targeting and activating promoters of endogenous genes. This approach worked for VEGF gene regulation in a stably transfected cell line (123). Rapamycin- or rapalog-inducible endogenous VEGF gene expression has been shown to be tight and rapid, of magnitude similar or superior to that achieved by the natural stimulus hypoxia, and accompanied by the simultaneous expression of the whole repertoire of VEGF splice variants. Although in its initial phase, this strategy has great potential for gene therapy (see also steroid-regulated systems).

The exclusive use of human components to assemble these two hybrid proteins should significantly decrease the possibility of immune response, but might not completely eliminate it. In fact, each polypeptide contains one or two novel junction epitopes that can be presented in the context of MHC class-I molecules and recognized as nonself, depending on the haplotype in which they are expressed. In theory, our greater knowledge of the immune system may permit these epitopes to be redesigned in order to reduce their possibility for presentation (124).

Of importance are the efforts of synthetic chemistry to expand the set of available heterodimerizers. Examples in this direction are the creation of fusions between methotrexate and a synthetic FKBP ligand or between methotrexate and dexamethasone, which are capable of triggering the formation

of heterodimerizers between DHFR and FKBP12 or between DHFR and the glucocorticoid receptor respectively (125,126). The extensive use of combinatorial chemistry, coupled with molecular modeling and protein design, will expand the number of reagents in the following years. We can expect to see a proliferation of dimerizer-regulated systems, which will significantly differ according to the type of inducer used. Demonstration of their feasibility in cell cultures will be merely the first and easiest step. This will have to be followed by the demonstration of suitable pharmacological properties and lack of toxic effects in vivo. As with every drug discovery project, these new chemical entities will have to pass the severe examination of in vivo pharmacology, safety assessment, and drug metabolism before becoming suitable for transferring to a clinical setting.

V. STEROID-REGULATED SYSTEMS

A. General Principles

Steroid receptors are natural examples of ligand-dependent transcription factors but cannot be used for gene therapy in their native form because they lack the required specificity, i.e., would also activate endogenous genes. Hence, by taking advantage of the modular nature of steroid receptors, in which DNA-binding, transactivation and ligand-binding functions reside in distinct domains, regulatory switches have been developed (127). Domain-swapping experiments have demonstrated the independent character of these domains. Heterologous proteins can be rendered hormone responsive by fusing them with the hormone-binding domain (HBD) of steroid receptors (128). In the absence of the ligand, the fusion protein is bound to the heat shock protein 90 complex (HSP90) through the HBD: in this state the fusion protein is inactive, probably due to steric hindrance and/or maintenance in an inactive conformation. Upon binding of the specific ligand to the HBD, the fusion protein is released from the HSP90 complex and becomes active.

The number of proteins adaptable to this approach is continuously expanding and includes kinases, recombinase, integrases, and oncogenes (129). This strategy has also been applied to generate hormone-dependent transcription factors. In pioneering works, the HBD of the estrogen receptor (ER) was fused to the DNA-binding domain of the yeast transcription factor GAL4 and to the Herpes simplex virus transactivation domain VP16 (GAL4-ER/HBD-VP16). This chimeric protein was able to stimulate transcription from artificial promoters containing GAL4-responsive elements in an Estradiol (E2)-dependent manner in cultured cells (130). This activator is predicted to be highly specific in terms of transgene transcription, because it only recognizes promoters containing the 17-mer GAL4 DNA-binding sequence that is not present in the mammalian genome. However, chimeric transactivators that carry natural HBDs of steroid receptors cannot be used in gene therapy applications because their activity would be severely influenced by endogenous steroids.

Thus, more recent studies have focused on the development of inducible chimeric activators equipped with mutant HBDs that do not interact with natural steroids but only bind to synthetic analogs. HBD-based chimeric transactivators that are specifically induced either by the antiprogestin RU486 or by the antiestrogen 4-hydroxytamoxifen (4-OHT) have been constructed and used to regulate transcription of target genes in animal models of gene therapy (Fig. 13). Here we describe the molecular architecture of these systems, summarize the results obtained in vivo, and discuss the potential of this type of switches for human gene therapy.

B. GeneSwitch: An RU486 Dependent Regulatory System

1. Transcription Factors

In 1992, BW. O'Malley and colleagues demonstrated that the C-terminus of the progesterone receptor (PR) is essential for its transcriptional activity upon interaction with progesterone (131). They also isolated a 42-amino acid C-terminal deletion mutant of the PR (called hPRB891) that no longer interacted with progesterone or other endogenous steroids, but retained its ability to bind progesterone antagonists, such as RU486, also known as mifepristone (Fig. 14) (131).

Based on this finding, the same group constructed a first version of an RU486-dependent transcription factor by fusing the HBD of hPRB891 (PR-HBD$_{640–891}$) C-terminally to the GAL4 DNA binding domain (DBD). This activator, called pGL, only minimally activated transcription from reporter genes cloned downstream of 4 tandem repeats of GAL4 binding sites (132). A more potent activator was obtained when the VP16 transactivation domain (residues 411–487) was fused N-terminally to the GAL4 DBD. This activator, called GLVP (or GS 1.0), enabled from 10- to 50-fold induction of reporter genes in cultured cells (132). As expected, GLVP transcriptional activity was stimulated only by adding RU486 but not in the presence of natural progesterone or synthetic progesterone agonists.

A more potent activator, called GL$_{914}$VPc' (or GS 2.0), was then generated by using a 19-amino-acid C-terminal deletion of the PR-HBD (aa 640–814) and by placing the VP16 activation domain at the C-terminus of the chimera (133). GS 2.0 induced gene expression in transiently transfected cells at 10-fold lower concentrations of RU486 than GS 1.0 (0.01 nM vs. 0.1 nM). However, GS 2.0 displayed a basal activity higher than that of GS 1.0, so that the net increase in induction was only 2–3 fold. By substituting the VP16 activation domain with that of the p65 subunit of human NF-κB protein, GLp65 activator (also called GS 3.0) was obtained, which was 2-fold less potent than GS 2.0, but displayed a lower basal activity in the uninduced state (78). Additional modifications were made by Valentis Inc., which now commercializes the system with the registered trademark ''GeneSwitch.'' The version called GLp 65.1 (or GS 3.1) is identical to GS 3.0 in terms of functionality and overall structure and only differs for the

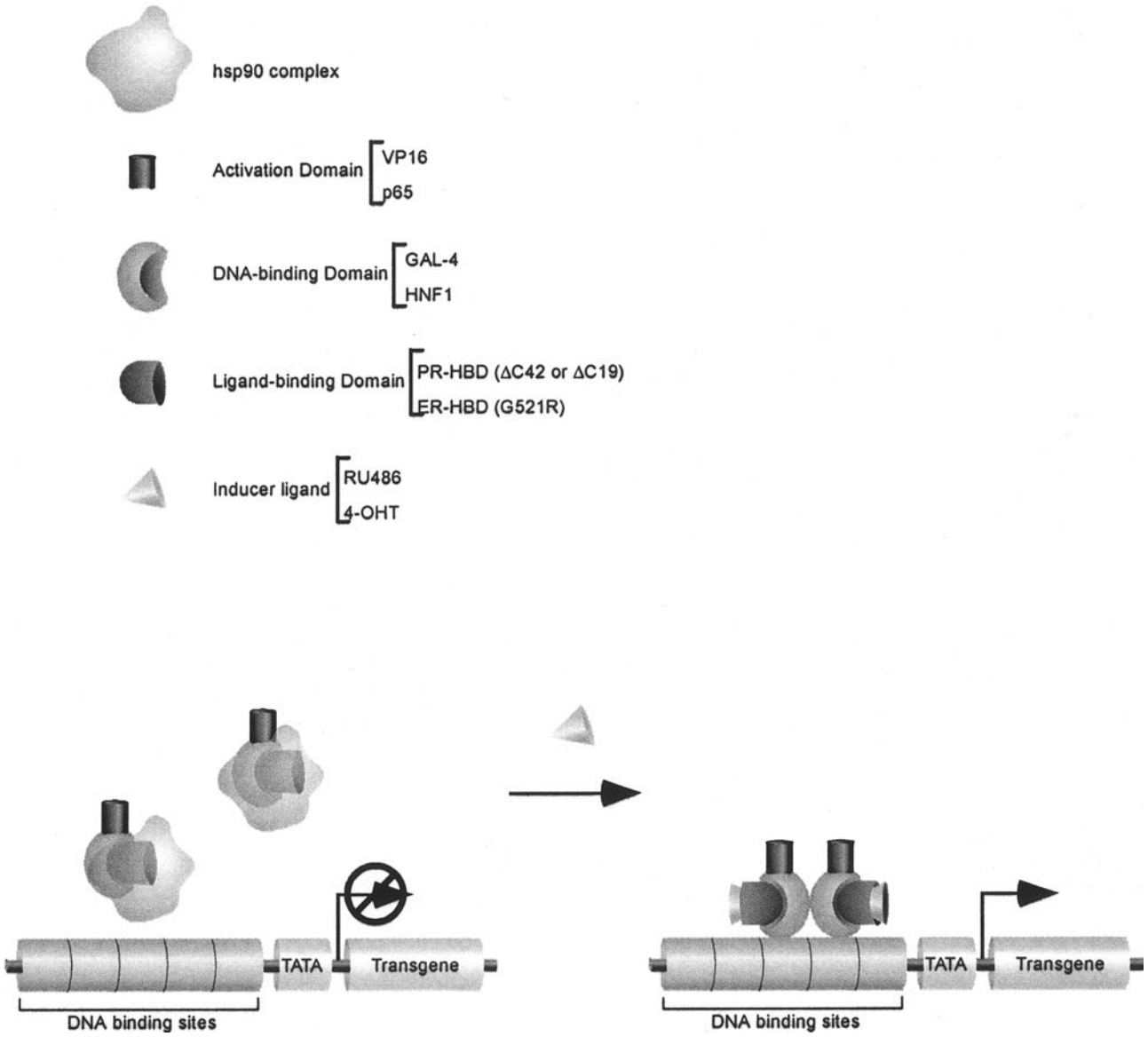

Figure 13 Hormone Binding Domain (HBD)-based regulatory switches. The activator consists of a DNA binding domain (GAL4 or HNF1, but also zinc fingers) fused to a mutant HBD, unable to bind endogenous steroids, and to an activation domain, such as VP16 and p65. In the absence of the ligand, the protein is bound to the hsp90 (heat-shock protein 90) complex and therefore inactive. In the presence of the inducer drug (RU486 or 4-OHT), the activator is first released from hsp90, and then binds and activates the target promoter, which consists of multimeric DNA binding sites upstream of a minimal promoter sequence.

shorter (3 aa long) linker between the PR-HBD and the p65 activation domain. GS 3.1 is responsive to RU486 concentrations ranging from 10–11 M to 10–8 M, with half-maximal activation occurring at 10–10 M (134). It is worth mentioning that a further version of this transactivator, called GS 4.0, has been described. It carries a shorter version of the GAL4 DBD and is claimed to have a lower basal activity as a consequence of the reduced capability to homodimerize in the absence of the ligand (135). However, no data have been published in support of this.

2. GeneSwitch Responsive Promoters

The structure of the GeneSwitch responsive promoters has not been significantly modified over the years (135). Initial experiments in cultured cells demonstrated that the composition of the minimal promoter cloned downstream of the mul-

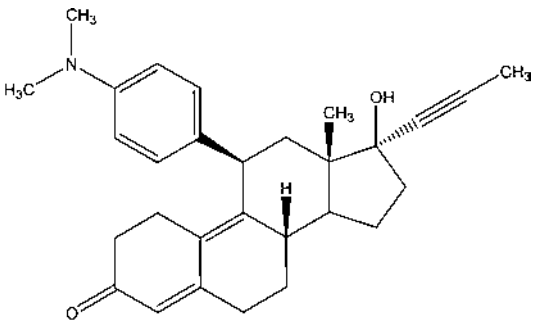

Figure 14 Chemical structure of RU486, inducer of the GeneSwitch system. RU486 (mifepristone) is a synthetic progesterone antagonist.

timeric GAL-binding sites significantly influences both the basal and maximal activity of the system. The minimal promoter that displayed the best compromise between basal activity and inducibility contained only the TATA box from the adenoviral major late promoter E1b. No further improvement in this direction has been reported following these early findings. In its most frequently used configuration, a GeneSwitch promoter consists of 6 tandem copies of GAL4 binding sites cloned upstream of the E1 TATA box (6 × GAL4-TATA)

3. GeneSwitch for Gene Therapy Applications

The efficiency of GeneSwitch in regulating transcription of target genes in vivo depends heavily on the delivery vectors used (Table 3). In a first study, a replication incompetent HSV vector carrying a CMV-driven GS 1.0 expression cassette and the β-gal gene under the control of a GAL responsive promoter was inoculated into rat hippocampus (136). Forty-eight hours postinjection, a 150-fold increased expression of β-gal was measured in brain extracts from rats treated with RU486. Minimal background β-gal expression was measured in untreated rats.

In another study using viral vectors, GS 3.0 under the control of the liver-specific transthyretin (TTR) promoter and a GAL4-responsive human growth hormone (hGH) cDNA were incorporated into the same helper-dependent adenoviral vector (78). Infection of cultured hepatoma cells and of mouse livers resulted in thousands-fold induction of hGH expression in rats treated with RU486. Following RU486 withdrawal, hGH expression returned to basal levels. hGH expression was repeatedly reinduced over a 9-month period, but the extent of induction progressively decreased (78,137). It was not established whether this decrease was caused by an immune response against hGH and/or GS 3.0, loss of vector DNA or transcriptional inactivation of TTR, and/or the GAL4-responsive promoter.

When delivered intramuscularly as plasmid DNA, GeneSwitch performed more poorly (Table 3). A 15-fold induction of SEAP gene expression was measured upon codelivery into mouse muscles of a CMV-driven GS 3.0 expression vector and a GAL4-responsive SEAP plasmid (134). Moreover, time-course experiments revealed that the leakiness increased over time, resulting in lower magnitude of induction at later time points. Interestingly, this poor induction level upon intramuscular delivery of plasmid DNA correlates with the finding that GeneSwitch displays a significant basal activity in transient transfection of several cell types, resulting in low induction ratios in the range of 10–40-fold (134). The relatively low potency of this system upon intramuscular plasmid injection was confirmed in experiments performed in rats (138).

Intramuscular delivery of GeneSwitch as a plasmid DNA may thus represent a valid therapeutic approach only in those cases where a low-fold induction is sufficient. This was demonstrated by a recent experiment in which intramuscular electroinjection of the GeneSwitch systems as plasmid DNA in SCID mice was used to regulate expression of the growth hormone-releasing hormone (GHRH) (Table 3). Prolonged treatment with RU486 led to a 1.1–1.7 rise in IGF-1 levels, presumably as a consequence of enhanced GHRH expression. This low-fold induction nonetheless provoked a significant increase in body weight, lean body mass, and bone mineral density with a concomitant decrease of fat mass (139).

In an attempt to increase the dynamic range of induction, an autoinducible GeneSwitch system was generated (140). In this system, the GS 3.1 transactivator was placed under the

Table 3 GeneSwitch System: In Vivo Studies in Animal Models of Gene Therapy

Transactivator	Transgene[a]	Vector	Tissue	Species	Refs.
GS 1.0	β-gal	HSV	Brain	rat	136
GS 3.0	GH	HD-Ad	Liver	mouse	78
GS 3.1	SEAP	Plasmid DNA	muscle	mouse	134
GS 3.1	SEAP, VEGF,	Plasmid DNA	muscle	mouse	140
GS 3.0	Epo	Plasmid DNA	muscle	rat	138
GS 4.0	GHRH	Plasmid DNA	muscle	mice	139

[a] β-gal = β-galactosidase; GH = Growth Hormone; SEAP = Secreted Alkaline Phosphatase; VEGF = Epo = erythropoietin; GHRH = Growth Hormone Releasing Hormone.

control of an autoinducible promoter that consists of four GAL4 sites upstream of the tk minimal promoter. Because of the residual basal activity of the tk minimal promoter (see above), GS 3.1 was expressed in the absence of the ligand, but only at low levels. Treatment with RU486 activates GS 3.1, which in turn promotes its own transcription, as well as that of the codelivered reporter gene cloned downstream of the classical 6 × GAL4-TATA promoter. In this autoinducible configuration, GeneSwitch displays lower basal activity, and the fold induction thus increases by one order of magnitude following intramuscular delivery in mice. However, expression levels of the target gene are lower than those obtained from more conventional constructs (140).

4. Inducer Drug

RU486 is a small, orally bioavailable, hydrophobic molecule that diffuses passively through cell membranes and freely distributes to several tissues, including the brain. RU486 is approved in several countries in combination with prostaglandins as a treatment to terminate pregnancy (141). In this context it is important to observe that RU486 dosages required to activate GeneSwitch are significantly lower than those required to induce abortion (132,134).

RU486 readily activates the GeneSwitch: in general, transgene product is already detectable 3 h after administration of a single dose. A dose-response experiment in mice demonstrated that GeneSwitch is maximally and half-maximally stimulated by intraperitoneal doses of about 300 μg/kg and 30 μg/kg, respectively (134). Since the bioavailability of RU486 after oral dosing is 40% in rodents, we can assume that GeneSwitch would be maximally stimulated by an oral dose of 600 μg/kg (142). A comparison between the pharmacokinetic properties of RU486 in rodents and humans allows the conclusion that maximal and half-maximal stimulation of the system in humans would be achieved by oral doses of approximately 30 mg and 3 mg, respectively (143). These dosages are significantly lower than those required to induce abortion (200–600 mg) and are well tolerated in humans (143). Nonetheless, even at these low dosages, RU486 affects the ovarian cycle and has a contraceptive activity related to the dosing schedule. For instance, a 2-mg daily dose inhibits ovulation, and 5 mg once weekly prevents implantation (144,145).

Finally, RU486 has been recently demonstrated to inactivate the human cytochrome P-405-3A4 (CYP3A4) and is thus expected to increase the bioavailability of several clinically used drugs that are metabolized by CYP-3A4 (146). The potential for drug-drug interaction must be taken into account in planning long-term treatment with RU486.

In conclusion, therefore, the contraceptive effect of RU486 at low doses but also the potential for drug-drug interactions may curtail the acceptance of GeneSwitch in clinical practice.

5. Perspectives

GeneSwitch has been constantly upgraded over the years in order to meet the stringent criteria required for gene therapy applications. Experiments in animals indicated that the system enables transgene regulation, but also suggested that additional improvements are required. A major drawback appears to be the ratio of potency vs. leakiness: GeneSwitch system is probably leakier and less potent than the most advanced versions of the tet system upon electroinjection of plasmids into mouse muscles. A direct comparison with the rapamycin-dependent system is not possible because the experimental settings in which the two systems have been so far tested are too different. The recently described autoregulatory circuit has reduced its leakiness but is also less potent and is not amenable for the construction of tissue-specific regulatory systems. In relation to this point, GS 4.0 may represent a major improvement.

The ligand-dependent activator has been partially humanized by using the p65 activation domain as a substitute for VP16: 82% of its amino acid sequence is now of human origin. Nonetheless, the GAL4 DBD may still be immunogenic in primates. In relation to this point, however, it is worth remembering that the system is highly flexible and can accept DBD of human origin (see below, 4-OHT-dependent regulatory system). Indeed, substituting the GAL4 DBD with artificial zinc fingers of predetermined specificity has generated novel RU486-inducible activators. In transient transfection experiments, these activators specifically induced transcription of reporter genes cloned downstream of multiple zinc-finger binding sites in an RU486-dependent manner (147,148).

C. HEA-3: A Humanized TAM-dependent Transcription Activator

1. General Principles

As outlined above, HBD-based chimeric activators have been generated, which carry synthetic polydactyl zinc fingers or the DBD of the yeast GAL4 transactivator. These DBDs enable specific transgene regulation because they recognize DNA sequences that are not the target of mammalian endogenous transcription factors. Nevertheless, these heterologous DBDs may be immunogenic in humans and therefore of limited efficacy for long-term gene therapy applications.

An HBD-based activator containing a DBD derived from a human protein is expected to have a lower potential for immunogenicity. DBDs derived from ubiquitous transcription factors are not appropriate for this purpose, because the responsive promoter would be constitutively activated in every tissue. In theory, however, customized molecular switches, which make use of human DBDs and effectively regulate gene expression in selected tissues, can be constructed. To this aim, two requirements must be fulfilled: first, the DBD must be derived from a transcription activator that is not expressed in the target cell/tissue; second, the inducible gene must be placed under the control of a synthetic promoter that carries only binding sites for that specific DBD. A fully humanized HBD-based activator (called HEA-3) specifically designed to work in muscle cells has been recently described and used to regulate gene expression in vivo (149).

2. Transcription Factor

HEA-3 is composed of three elements. At the N-terminus, it carries the DBD of the transcription factor Hepatocyte Nuclear Factor 1 (HNF-1). HNF-1α and its closely related variant HNF-1β, are dimeric homeoproteins expressed mainly in hepatocytes, where they are required for the expression of liver specific genes, and in a few other epithelial cells (150,151). The two proteins are not expressed in muscle (152). The DBD of human HNF-1 was fused in frame with the HBD of the human ERα. To prevent activation by endogenous steroids, an HBD containing the mutation glycine to arginine at residue 521 (G521R) was used. This mutant HBD binds the antiestrogen 4-OHT, whose structure is shown in Fig. 15, but not endogenous estradiol (E_2) (153,154). The activation domain from human p65 protein was added at the C-terminus (78).

3. Target Promoter

As for other regulatory switches, HEA-3 responsive promoters consist of multimeric binding sites cloned upstream of a minimal promoter. Seven tandem repeats of HNF1 binding sites were linked either to a minimal promoter sequence derived from the human C-Reactive Protein (CRP) gene (7 × H1/CRP promoter) or to the TATA box sequence from the adenovirus E1b gene (7 × H1/E1b promoter) (149).

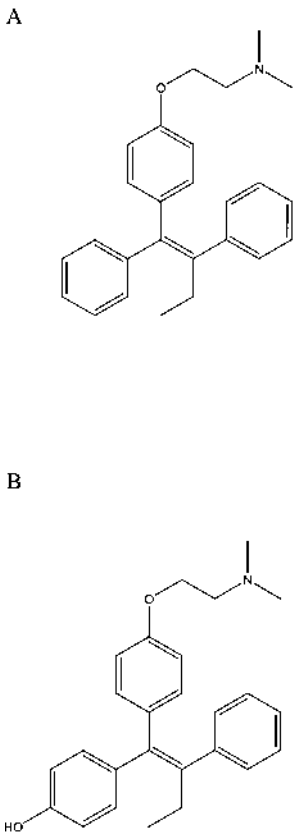

Figure 15 Chemical structure of Tamoxifen (TAM) and 4-hydroxytamoxifen (4-OHT), inducers of HEA-3. TAM (A) and 4-OHT (B) activates the HEA-3-based system in vivo and in vitro, respectively. See text for additional details.

4. HEA-3 for Gene Therapy Applications

Artificial promoters containing multimeric HNF1 binding sites were silent in muscles and in cell lines that lacked endogenous HNF1α but were strongly activated by HEA-3 in the presence of 4-OHT. The system was half-maximally stimulated at a 4-OHT concentration of about 40 nM. In transiently transfected cells, HEA-3 induced thousands-fold induction of reporter genes in a 4-OHT-dependent manner. Importantly, E2, progesterone, and RU486 did not stimulate the system activators (149).

The system was delivered in mice as plasmid DNA by using muscle electroinjection and induced by oral doses of Tamoxifen (TAM, Fig. 15), which is converted to the active metabolite 4-OHT in vivo. In this experimental setting, HEA-3 upregulated by about 100-fold SEAP gene expression in a strictly TAM-dependent manner. Dose response experiments demonstrated that the system was significantly induced by as low as 1 mg/kg of TAM. HEA-3 also enabled long-term and fully reversible regulation of mouse erythropoietin gene over a 10-month period (149).

5. Inducer Drug: Tamoxifen

Tamoxifen is a clinically validated drug that is currently used to prevent recurrence of breast cancer (155). However, several lines of evidence suggest that the system should be significantly improved in order to meet the safety requirements required for applications in humans.

An oral dose of 1 mg/kg/day of TAM significantly (but not maximally) stimulates HEA-3 in mice (149). This dosage regimen ensures a steady–state 4-OHT plasma concentration in mice of about 30 nM, a value close to the EC_{50} of the drug for HEA-3 (see above). The plasma concentration of 4-OHT in patients undergoing long-term TAM therapy (20 mg/kg, daily) is about 10 nM: this indicates that TAM dosages higher than those normally used in clinical practice would be required in humans to exploit the full dynamic range of the HEA-3-based system (155–158). Therefore, the sensitivity of this system should be increased by at least one order of magnitude for gene therapy applications in humans. This is of particular relevance when considering that TAM is extremely well tolerated at the usual dosage, but that prolonged treatment (i.e., years) is associated with a low, but well-defined, increased risk of endometrial cancer (157,159).

6. Perspectives

HEA-3 was specifically designed to work in muscle, as the use of this tissue as a bioreactor for producing therapeutic proteins is an attractive possibility for gene therapy (160). However, HEA-3 can be considered a prototype of a series of humanized regulatory switches. DBDs of transcription factors other than that of HNF1 can thus be selected to construct

additional humanized chimeras specifically designed to work in tissues other than muscle. It is tempting to speculate that the combined use of tissue-specific DBDs and of mutant HBDs recognizing only synthetic steroids would generate a whole series of ''customized,'' ligand-dependent, humanized chimeras, specifically designed to work in a restricted range of tissues and cells.

Such customized molecular switches would recognize DNA sites within the chromosomes and might therefore affect endogenous gene expression. This may or may not be a concern, depending of the affected genes. However, it is worth considering that gene transcription in higher eukaryotes is governed by a complex network of interacting activating and repressing factors, whose equilibrium in a specific cell cannot be easily modified by overexpressing a single transcription factor not normally present (161). Therefore, we can hypothesize that molecular switches such as HEA-3 are unlikely to profoundly modify the expression profile of a target cell. This is partially confirmed by the observation that both albumin and α1-AT genes, whose expression in hepatocytes is regulated by HNF1, are not turned on in HEA-3-transduced muscles (149). Nonetheless, it will be necessary to study this safety issue in greater detail with the help of new sensitive technologies such as DNA microarray or whole-cell proteomics.

HEA-3 uses tamoxifen, a clinically accepted drug that acts as a steroid receptor antagonist. For gene therapy applications, inducer drugs that do not have any effect on endogenous proteins would be more appropriate. Future work should focus on redesigning the HBD-hormone interface in order to differentiate it from endogenous receptors, and to identify novel ligands that only bind to those mutated HBDs, an approach that has been successfully used to generate nonimmunosuppressive analogs of rapamycin or FK506.

VI. ECDYSONE-RESPONSIVE REGULATORY SYSTEM

A. General Principles

The steroid hormone ecdysone, which is normally found in mammals, plays a fundamental role during *Drosophila* molting and metamorphosis. Pulses of 20-hydroxyecdysone mediate that cascade of morphological modification, which results in degeneration of larval tissues and development of adult structures (162). Ecdysone acts by stimulating a transcription factor, the ecdysone receptor (EcR). The EcR is a member of the nuclear receptor superfamily, and its modular organization resembles that of steroid receptors (163). The functional EcR is a noncovalent heterodimer with the insect protein ultraspiracle (USP): the two proteins share the multidomain organization of the nuclear receptor family. USP is an obligatory heterodimeric partner of RcR, required for both ligand and DNA binding (164). EcR carries a transcriptional silencer in its ligand-binding domain, which acts by recruiting corepressors such as N-CoR and SMRT (165,166). Binding of the cognate ligand induces a conformational change that makes the corepressor dissociate from the receptor and recruits activators, which ultimately leads to transcriptional activation of genes under EcR control. Also, USP contains a ligand-binding domain, but the natural ligand is unknown: interestingly, recent evidence suggests the USP ligand-binding pocket may be locked in an inactive conformation (167,168).

The mammalian counterpart of USP is the retinoid-X-receptor (RxR), a member of the nuclear receptor family, whose natural ligand is the 9-*cis*-retinoic acid (9cRA), a vitamin A metabolite, which binds to and activates RxR (169). RxR forms homodimers as well as heterodimers with several hormone and orphan receptors. The vitamin D receptor (VDR), the peroxisome proliferator activated receptor gamma (PPAR-γ), the hepatocyte nuclear factor 4 (HNF4), the retinoic acid receptor (RAR) and the thyroid hormone receptor (TR) are among the normal heterodimeric partners of RxR (169). RxR heterodimerize also with the EcR, albeit much less efficiently than USP (170,171).

B. Transcription Factors

In pioneering experiments, insect responsiveness to ecdysone or its analog muristerone A (Fig. 16), was recreated in cells by cotransfecting Drosophila EcR and USP with an EcR-responsive gene. However, in this configuration, muristerone A stimulated transgene expression only to a very limited extent (164,172).

Several modifications were introduced to enhance the dynamic range of the system generating a system schematically represented on Fig. 17. The N-terminal activation domain of EcR was substituted with the VP16 activation domain, thus obtaining a chimeric activator called VpEcR, and the natural heterodimeric partner USP was substituted with RxR. This heterodimer stimulated gene expression up to 1000-fold in cultured CV1 cells (173). Since the mammalian farnesoid-X-receptor (FxR) can weakly activate transcription from EcR-responsive elements, using a mutant ECR DBD capable of binding a synthetic responsive element further increased the specificity of gene regulation. The mutant activator and the synthetic sequence were called VgEcR and E/GRE element, respectively (173). Although FxR can activate a promoter containing the wild-type EcR element, it cannot bind the E/GRE site. The salient attribute of this system was its low basal activity, which is probably due to the natural repressing capability of the EcR (see above) in the absence of the ligand. The VgEcR/rRxR system is now sold commercially and is widely for conditional gene expression in transfected cells.

A modified system, called RheoSwitch, has been recently described. It relies on the coexpression in target cells of GAL4-EcR, which consists of the fusion of the GAL DBD with the EcR of the lepidopteran Choristoneura occidentalis, and VP16-RxR, a fusion between RxR and the VP16 activation domain (174). In stable transfectants, RheoSwitch enabled up to 500- and 100-fold induction of integrated SEAP and luciferase genes, respectively. In this case, GS-E was used as an inducer (Fig. 16). GS-E is a member of a family of diacylhydrazines

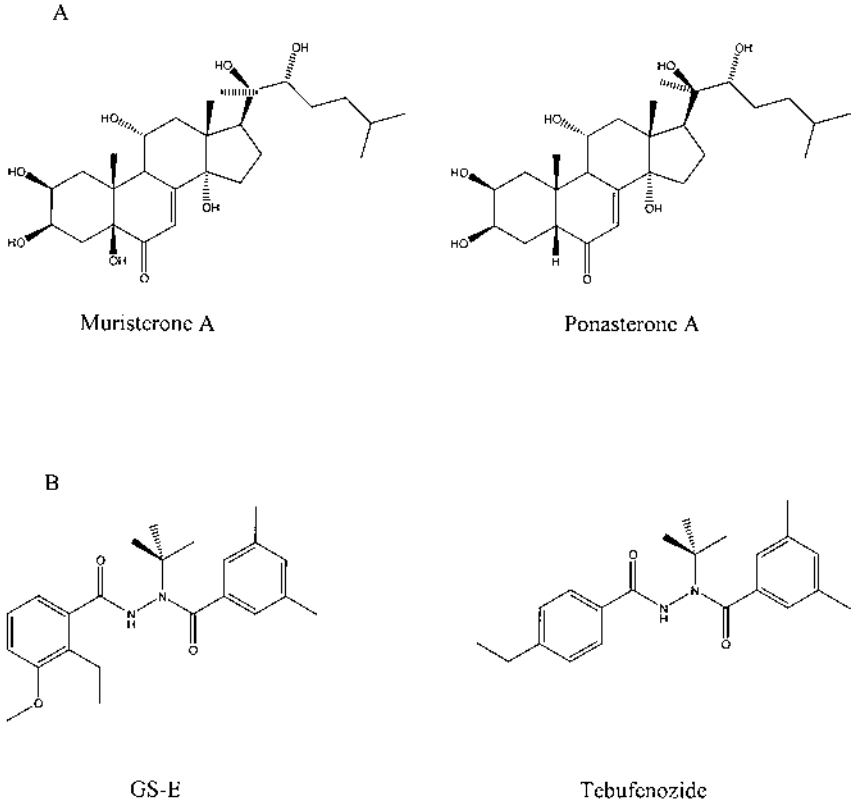

Figure 16 Chemical structure of ecdysone-dependent regulatory system inducers. (A) Plant-derived inducers (Muristerone A and Ponasterone A); (B) synthetic dibenzoylhydrazine compounds (GS-E, Tebufenozide).

that have been found to act as nonsteroidal ecdysone mimics that can function as gene inducers (see below).

VgEcR/rRxR and RheoSwitch systems are based on the Drosophila EcR, which is a reluctant dimer partner for RxR. Since RxR is generally expressed at low levels, efficient EcR mediated stimulation can only be achieved by overexpressing RxR in the target cells (164,172). Overexpression of RxR in target cells is undesirable for human gene therapy (see next paragraph). Therefore, the recent finding that EcR from Bombyx mori (BeCR) dimerize much more efficiently with RxR is of particular interest (175). The determinants of this higher affinity map within the HBD and the hinge D domain of BEcR. As a consequence of this higher affinity dimerization, VBEcR (a fusion between BEcR and the VP16 activation domain) strongly stimulates transcription in the absence of cotransfected RxR. BecR is also more responsive than EcR to nonsteroidal agonists (175).

In a more advanced version, the properties of VgEcR and of BEcR were combined. The activation and DNA-binding domain of VgEcR were fused to the hinge region and the HBD of BEcR. This hybrid-receptor (called DB-EcR) thus recognizes the unnatural E/GRE responsive elements but does not require RxR to induce gene expression in mammalian cells and responds more efficiently to nonsteroidal agonists (176).

C. Responsive Promoters

Various EcR responsive promoters have been described that shares the same basic architecture. They usually consist of four EcR-responsive elements (or E/GRE sites, in the case of VgEcR and DB-EcR) cloned upstream of a variety of minimal promoters such as those derived from the Drosophila heat shock or timidine kinase genes (173,175). RheoSwitch recognizes multimeric GAL4 binding sites (174).

D. Ecdysone-regulated Systems for Gene Therapy Applications

The ecdysone system enables tight regulation of gene expression in cell culture and transgenic animals, but its potential for gene therapy applications has not been systematically explored yet.

As to the VgEcR/rRxR and RheoSwitch, several shortcomings may prevent their application in human gene therapy. Both systems are based on overexpression of wild-type RxR, which would thus be overrepresented in all endogenous RxR-containing receptors (see above). This poses a safety concern, considering the large number of metabolic pathways in which heterodimers containing RxR are involved. The recent finding

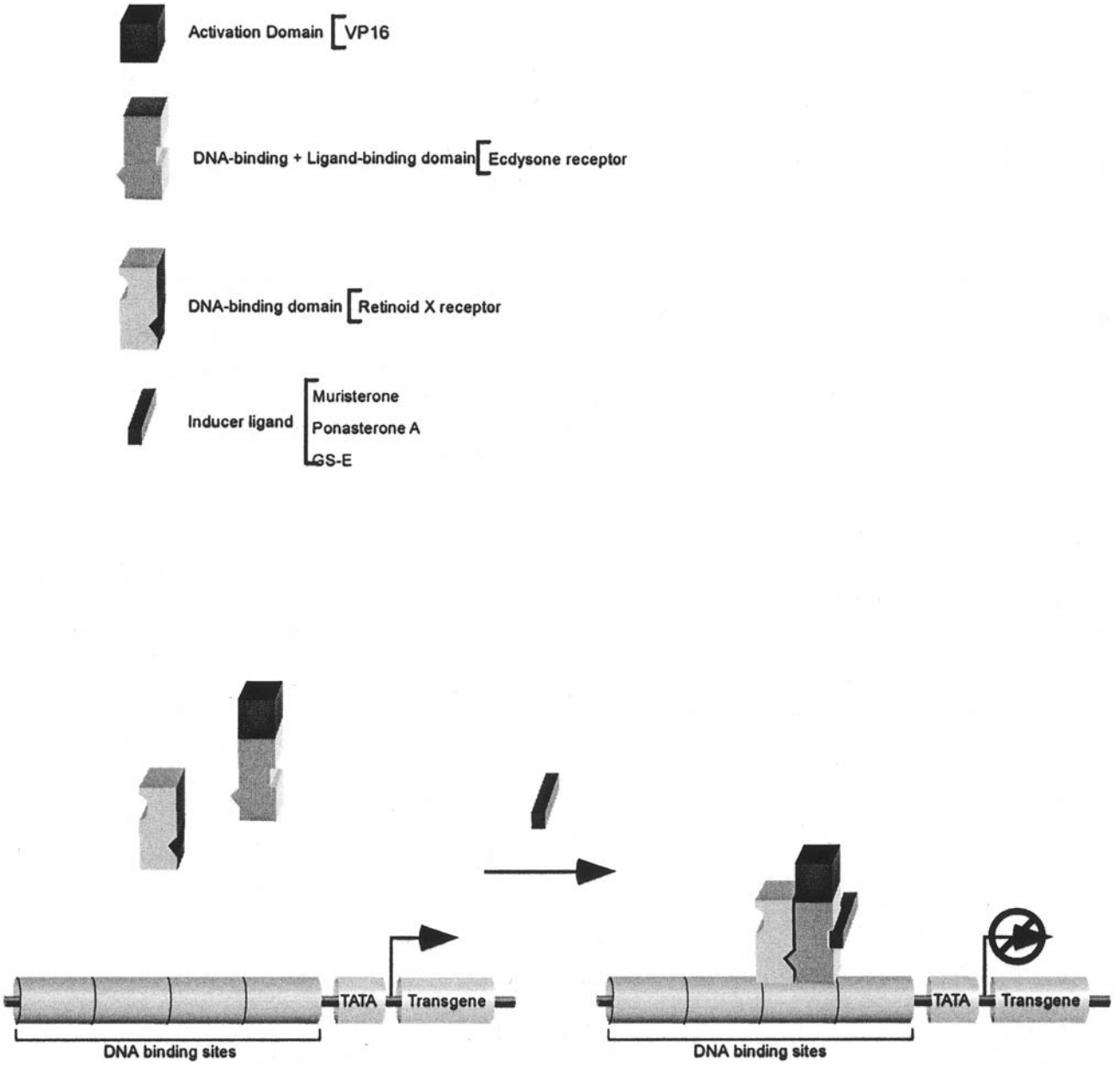

Figure 17 Ecdysone-responsive regulatory system. One component consists of the human retinoid X receptor (RxR) that carries a DNA-binding domain. The second component consists of a modified ecdysone receptor (EcR) that carries a DNA binding domain and is fused to a VP16 activation domain. Following administration of the drug, the modified EcR binds the ligand and forms a functional heterodimer with RxR. This heterodimer recognizes a specific DNA sequence and therefore activates promoters containing multimers of this sequence.

that RxR overexpression in cardiomyocyte caused dilated cardiomyopathy in mice further emphasizes this aspect (177). Similar concerns would also apply to USP, with the additional problem that this heterologous protein might prove strongly immunogenic in humans. Another issue is the fact that ligands for RxR can modulate the effect of EcR agonists on the RxR/EcR heterodimers (171). This implies that activity of the RxR/EcR receptor would not be controlled solely by the inducer drug but also by endogenous natural ligands for RxR, such as the natural agonist 9cRA. Notably, mutant RxR unable to

bind the endogenous ligands cannot be used because they would probably also affect activity of all the heterodimeric receptor for RxR.

As outlined in the preceding paragraph, DB-EcR does not require a codelivered dimerizing partner: its use is thus much more suitable for gene therapy applications. So far, this system has been delivered in vivo only by intramyocardial injection of recombinant adenovirus vectors in adult rats. In a first study, the VgEcR/rRxR and DB-EcR regulatory systems were inserted into two distinct Ad vectors (176). Each vector was then coinjected intramyocardially with a reporter Ad vector containing a luciferase gene under the control of an E/GRE-based promoter: rats were then injected intraperitoneally with 45 mg of GS-E (Fig. 16). Three days after, relative luciferase activity was measured: GS-E treatment caused a 40-fold induction of luciferase activity in rats injected with the DB-EcR regulatory system, while it was essentially ineffective in those injected with VgEcR/rRxR. This confirms that VgEcR/rRxR is inefficiently activated by GS-E-like molecules. Basal activities of the two systems were comparably low (176). In a related study, the DB-EcR regulatory system was delivered by adenoviral vectors to express a dominant negative form of a myocardial ion channel in a GS-E-dependent manner (178).

These studies indicate that DB-EcR may be useful for gene therapy applications, but they also highlight that the potency of the system is at this stage inadequate for most gene therapy applications and should therefore be increased. Moreover, the sequestration of endogenous RxR by DB-EcR could have deleterious effects, which may only become evident in long-term experiments. Additional work with different vectors and target tissues is required.

Finally, the presence of the nonhuman protein domains poses the usual concerns about the potential immunogenicity.

E. Inducer Drugs

Ecdysterone is not able to efficiently stimulate the ecdysone-responsive system in mammalian cells (171). More efficient analogs were isolated from plants, which protect themselves from insect feeding by producing substances that are toxic to insects. Ecdysteroids are among these chemicals: when insect larvae eat the leaves of the plant, they also ingest ecdysteroids, which stimulate the EcR to start an abnormal molting that leads to premature death (179). Ecdysteroids may represent up to 1% of the total dry weight of a plant (180).

The most efficient phytoecdysteroids isolated so far are Muristerone A (murA) and Ponasterone A (PonA) (Fig. 16). MurA was isolated in the early 1970s from the seeds of *kaladana*, a rare plant native to the southern slopes of the Himalayas. It proved capable of stimulating the VgEcR/rRxR and related systems with an EC_{50} comprised between 0.5 and 1 uM (173). Difficulties in obtaining seeds from *kaladana* prompted investigators to identify inducers derived from other plants. Ponasterone A (ponA) was thus identified: it can be purified from the leaves of widespread diffused plants and is as potent as MurA (181). It is conceivable that other inducers will be isolated in the future.

Ecdysteroids are usually presented as safe compounds based on the consideration that humans eat large amounts of phytoecdysteroids (contained in vegetables) without apparent detrimental effect (180). Nonetheless, it is clear that in the light of their potential use in human gene therapy, more detailed toxicology data would be required. Oral bioavailability of ecdysteroids is also a matter of concern: PonA and MurA have always been administered to rodents by intraperitoneal or subcutaneous injections (173,176,178,181). The possibility also exists that patients harboring the ecdysone-receptor switch will have to reduce their oral intake of dietary phytoecdysteroids to avoid nonspecific activation of the system.

A family of bisacylhydrazines has been identified that function as insecticides by mimicking the activity of ecdysone on the EcR (179). Not surprisingly, they have also been found to function as inducers of the ecdysoneswitch. These molecules, such as tebufenozide and GS-E, are less active than PonA on the VgEcR/rRxR system (Fig. 16) (181), but more potent on the DB-EcR switch (176). These compounds can be easily manufactured but show a very poor solubility: this limits their potential for in vivo applications (179,181). A recent study demonstrates that molecular modeling and site directed mutagenesis of the EcR generated a mutant EcR, which induces transcription of target genes in response to bisacylhydrazines but not to ecdysteroids (182). As outlined above, because of the dietary intake, the development of ecdysteroid-insensitive regulatory switches might be necessary in humans. Therefore, albeit the clinical potential of bisacylhydrazines remains to be established, more soluble molecules would represent an important starting point and prove a useful tool.

VII. CONCLUSIONS

The generation and refinement of new ligand-induced transcription switches is an area of active investigation, but probably none of the available systems is ready for use in humans. Much progress has been made during the last few years to improve performances of tetracycline, rapamycin, RU486, and ecdysone technologies in terms of tightness of regulation and inducibility. It can be predicted that also the most recently described systems will undergo a similar process of optimization. However, while constructing systems that work in cell cultures and animal models is becoming a relatively easy task, the generation of a transcription switch compatible with long-term application in humans is still a very difficult challenge. In a very schematic view, the development of a ligand-induced transcription switch for use in humans is mainly an immunological and a medicinal chemistry/pharmacology task. The activators must not be recognized as nonself, and the ligand must have good oral bioavailability and pharmacokinetic profiles in the absence of toxicity. The need to satisfy both requirements tremendously complicates this particular process and reduces the probability of success.

This is clearly evident if we compare the tetracycline and dimerizer technologies that emerge over the others for their

better characterization in living organisms, including nonhuman primates. On one side, there is the tetracycline system that relies on proteins of bacterial origin that may be immunogenic in humans but is induced by a relatively safe drug. On the other side stands the dimerizer-based switch, which has a reduced potential for immunogenicity but is induced by non-immunosuppressive rapamycin analogs that have not yet been characterized for their pharmacological and safety profile.

Despite these difficulties, the future is still bright with promise: the enormous progress in the last years makes us confident that systems fully compatible with use in humans will emerge in a not-too-distant future.

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23

Monitoring Gene Therapy in Living Subjects with Molecular Imaging Technologies

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I. INTRODUCTION

Human gene therapy trials can be significantly aided by the ability to determine the location(s), magnitude, and time variation of transgene expression. Gene therapy vectors have to be held accountable for their actions, and recent developments in noninvasively imaging gene expression in living subjects is certain to help in this regard. Following delivery of a therapeutic vector into a patient, a gene therapist wants to know the answers to some fundamental questions: Did the vector get delivered to the desired target organ, tumor, or cell population, or have inappropriate tissues been transfected? Did gene transfer and/or transcription take place? How much gene product is being generated from the transgene? How much control do I have in the expression of this vector? What happens to expression levels as the patient responds (or fails) to therapy? Current clinical methods have difficulty in answering these questions: they rely upon serum markers, tissue-sampling followed by histochemical analysis or autoradiography, anatomical-based imaging, and physical exam, most of which are either inefficient, invasive, or inadequate in this era of targeted gene therapy and molecular medicine. Answering these questions rapidly, easily, and effectively during a patient's clinical course is clearly desirable, not only to tailor an individual patient's needs and improve clinical outcomes, but also to promote gene therapy for routine clinical use.

Recognizing this need, molecular imaging researchers have advanced the principles of reporter gene technology for use in intact, viable subjects. To the molecular biologists, reporter gene technology is well-trodden territory, and they have been familiar with the process of coupling a reporter gene to a therapeutic gene as a means of measuring the efficacy of gene delivery and tracking gene expression. Commonly used reporter genes for in vitro or ex vivo analyses include: a) chloramphenicol acetyltransferase, which transfers (radioactive) acetyl groups to chloramphenicol. Detection is rendered through thin-layer chromatography and autoradiography (1–3); b) β-galactosidase (GAL), which hydrolyzes colorless galactosides to yield colored precipitate, is detected via tissue extracts or histochemical techniques (4–6); c) β-lactamase, which catalyzes hydrolysis of cephalosporin, is monitored by a change in fluorescence emission of a substrate (7,8); d) green fluorescent protein (GFP), which fluoresces upon radiation, is examined with epifluorescence microscopy or utilized in cell sorting techniques (9–11); e) firefly luciferase, which oxidizes D-Luciferin to generate bioluminescence, can be visualized using luminometry or microscopes outfitted to specialized cameras (12,13); and f) *Renilla* luciferase, which catalyzes oxidation of coelenterazine, leading to bioluminescence (14). While some of these technologies cannot make the jump into studies of living subjects (e.g., chloramphenicol acetyltransferase, β-galactosidase) because of technical limitations, the optically-based technologies, such as those based on GFP and the luciferases, have been applied to intact, living subjects using sensitive optical detection systems (15–18).

Furthermore, there has been an exciting surge in the development of reporter gene technologies that are compatible with current clinical imaging modalities such as the radionuclide-based techniques [positron emission tomography (PET) and single photon emissions tomography (SPECT)], magnetic resonance imaging (MRI), and magnetic resonance spectroscopy (MRS). To understand these clinical technologies does not

require a large leap of faith. In fact, they are conceptually similar to the optical systems described above. All in vivo reporter techniques, including the aforementioned optical techniques, depend on the detection of electromagnetic homing signals that arise either from the probe itself or the specific interaction of a reporter protein and its corresponding reporter probe. Just as GFP emits photons in the visible range of electromagnetic spectrum that can be seen with the naked eye or cameras, radionuclide-based reporter techniques emit photons in the gamma ray-wave range that are detected by specialized scintillation crystals. Table 1 lists the various reporter systems available for in vivo imaging. In the following review, we will discuss the basic principles of the various tools now available, display several examples of the images generated from such tools, and, at the end, provide a strategy to help those scientists or gene therapists interested in using in vivo reporter techniques to choose optimal strategies.

II. IMAGING INSTRUMENTATION FOR LIVING SUBJECTS

A. Basic Principles of Optical Charged Couple Device (CCD) Imaging (Fluorescence and Bioluminescence)

Although photons emitted in the visible light range of the electromagnetic spectrum face considerable obstacles traveling through layers of tissues, notable advances in light sensor technology have permitted the use of optical reporter genes in intact organisms. There are fundamentally 2 different types of optically based imaging systems: a) fluorescence imaging, which uses emitters such as green fluorescent protein (GFP), wavelength-shifted GFP mutants, red fluorescent protein (RFP), ''smart'' near-infrared fluorescent (NIRF) probes, and b) bioluminescence imaging, which utilizes systems such as Firefly luciferase-D-Luciferin or Renilla luciferase-coelenterazine (15,19,20). Each of these systems will be discussed in further detail in a later section. Emission of light from fluorescent markers requires external light excitation while bioluminescence systems generate light de novo after an injectable substrate is introduced and the appropriate conditions are met (Fig. 1). In both cases, light emitted from either system can be detected with a thermoelectrically cooled charge-couple device camera (CCD) since they emit light in the visible light range (400 nm to 750 nm) to near-infrared range (~800 nm). Cooled to −120 to −150°C, these cameras can detect weakly luminescent sources within a light-tight chamber. Being exquisitely sensitive to light, these desktop camera systems allow for quantitative analysis of the data. The method of imaging bioluminescence sources in living subjects with a CCD camera is relatively straightforward: the animal is anesthetized, subsequently injected intravenously or intraperitoneally with the substrate and placed in the light-tight chamber for a few seconds to minutes. A standard light photographic image of the animal is obtained, followed by a bioluminescence image captured by the cooled CCD camera positioned above the subject within the confines of the dark chamber. A computer subsequently superimposes the 2 images on one another, and relative location of luciferase activity is inferred from the composite image. An adjacent color scale quantitates relative or absolute number of photons detected. This scale does not reflect the color (wavelength) of the emitted photons, but only the number of such photons, measured in relative light units per minute (RLU/min). Differences between fluorescence and bioluminescence systems are discussed in a later section of this chapter.

Comparison of optical-based imaging systems with the other imaging modalities, such as the radionuclide-based or MRI-based systems, reveals important differences. Advantages of optically-based reporter systems is that they are at least an order of magnitude more sensitive than the radionuclide-based techniques at limited depths (21). Furthermore, the direct and indirect costs are generally less than radionuclide-based techniques or MRI. However, there is significantly less spatial information obtained from optical imaging, and signal obtained from light-emitting reporter systems is limited by the tissue depth from which it arises. Furthermore, while significant progress has been made to localize fluorescent signals tomographically to obtain distribution of fluorochromes in deep tissues (22), there is currently no commercially available technique to obtain 3-dimensional localization of the targeted optical probes.

B. Basic Principles of PET

With recent breakthroughs in molecular/cell biology and target discovery, it is now possible to design specific markers to image events noninvasively in small animals and humans with positron emission tomography (PET) (23,24). Natural biological molecules such as glucose, peptides, proteins, and a variety of other structures can either be labeled with a radioisotope or slightly modified to accommodate a radioisotope. In the jargon of molecular imagers, these radiolabeled molecules are referred to as molecular probes, reporter probes, markers, or tracers. In PET imaging, molecules are labeled with isotopes that emit positrons from their nucleus. This is in contrast to gamma camera or SPECT imaging (discussed below), where molecules are ''tagged'' with radioisotopes that emit gamma rays. When a molecular probe is injected into a living subject and then placed into a PET scanner, the image we acquire is a snapshot of the physiological distribution and concentration of that tracer.

Let's take the example of 2-deoxy-2-[^{18}F] fluoro-D-deoxyglucose (FDG), a glucose analog labeled with the positron-emitting isotope, ^{18}F. In the synthesis of this tracer, PET radiochemists have devised a method to replace one of the hydrogens with the ^{18}F radioisotope. Following intravenous injection, this particular probe distributes readily from the intravascular compartments to the extravascular and intracellular compartments. FDG becomes sequestered in cellular populations and tissues that have a predilection for glucose, i.e., cells that possess larger number of glucose transporters and/or hexokinase II activity. When FDG encounters hexokinase II, it becomes phosphorylated and subsequently trapped

Table 1 Summary of Reporter Gene Systems Used in Living Subjects

Method	Imaging	Reporter gene	Action	Imaging agent/substrate	Ref.
In vitro (selected few)	Chromatography and autoradiography	Chloramphenicol acetyltransferase	Acetylation	Radioactive acetyl group	(2)
	Light Microscopy	β-galactosidase	Hydrolysis	Metabolized galactosides	(6)
		β-glucuronidase	Hydrolysis	Metabolized glucuronides	(109)
	Epifluorescence Microscopy	β-lactamase	Hydrolysis	Metabolized cephalosporin	(8)
		Green fluorescent protein	Light excitation	None (irradiation)	(9)
		Red fluorescent protein	Light excitation	None (irradiation)	(42)
		Enhanced Green Fluorescent protein	Light excitation	None (irradiation)	(37)
	MRI	Tyrosinase	Hydroxylation	Melanin production	(110)
Living subjects	Optical: Fluorescence	Green fluorescent protein	Light excitation	None (irradiation)	(18)
		Red fluorescent protein (dsRed)	Light excitation	None (irradiation)	(111)
	Optical: Fluorescence activation	Cathepsin D	Enzymatic cleavage	Quenched NIRF fluorochromes	(19)
		Matrix metalloproteinase (MMP2)	Enzymatic cleavage	Quenched NIRF fluorochromes	(44)
	Optical: Bioluminescence	Luciferase (Firefly)	Luciferase-D-Luciferin action in presence of ATP, Mg^{2+} and O_2	D-Luciferin	(35)
		Luciferase (Renilla)	Luciferase-D-Luciferin action in presence of O_2 only.	D-Luciferin Coelentcrazine	(15)
	Radionuclide Imaging: PET	Herpes simplex virus type I thymidine kinase (HSV1-*tk*)	Phosphorylation	[^{124}I]FIAU [^{18}F]GCV [^{18}F]PCV [^{18}F]FHPG [^{18}F]FHBG	(112)
		Mutant HSV1-*tk* (HSV1-sr39-*tk*)	Phosporylation	[^{18}F]PCV [^{18}F]FHBG	(60)
		Cytosine deaminase	Enzymatic conversion	[^{18}F]-5-fluorocytosine	(66)
		L-amino acid decarboxylase (AADC)	Enzymatic conversion	6-[^{18}F]fluoro-L-*m*-tyrosine	(113)
		Dopamine-2-receptor (D2R)	Receptor-ligand	[^{18}F]FESP	(49)
		Mutant D2R	Receptor-ligand	[^{18}F]FESP	(48)
		Sodiom/Iodide Symporter (NIS)	Ion pump	^{124}I	(65)
	Radionuclide Imaging: Gamma camcra/SPECT	Somatostatin receptor	Affinity binding	[^{99m}Tc]Somatostatin analog P2045 [^{111}In]DTPA-d-Phe-octreotide [^{188}Re]Somatostatin analog	(76)
		HSV1-*tk*	Phosphorylation	[^{131}I]FJAU [^{125}I]FIAU	(76) (114)
		Sodium/Iodide Symporter (NIS)	Ion pump	^{123}I, ^{131}I, ^{99m}Tc Pertechnetate	(63)
		Gastrin-releasing peptide receptor	Receptor-ligand	[^{125}I]mIP-Des-Met^{14}-bombesin (7–13)NH_2 [^{125}I]Bombesin [^{99m}Tc]Bombesin analogue	(115)
	MRI	β-galactosidase	Hydrolysis	EgadMe	(82)
		Engineered transferrin receptor	Receptor-ligand internalization	Tf-MION Tf-CLIO	(79) (81)
	MRS	Creatine kinase	Chemical shift spectroscopy	Phosphocreatine	(83)
		Arginine kinase	Chemical shift spectroscopy	Phosphoarginine	(84)
		Cytosine deaminase	Spectroscopy	Production of 5-fluorouracil (5-FU)	(66)

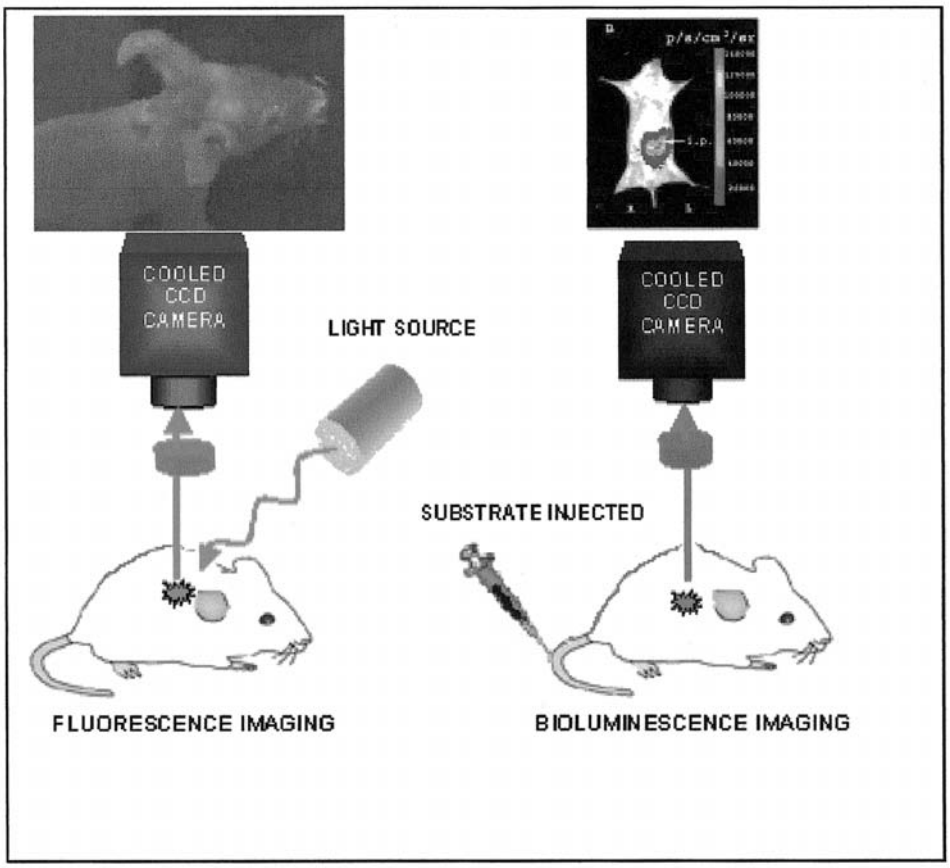

Figure 1 Basic Principles of Optical CCD Imaging (Fluorescence/Bioluminescence) There are fundamentally 2 different types of optically-based imaging systems: fluorescence imaging, which uses emitters such as green fluorescent protein (GFP), wavelength-shifted GFP mutants, red fluorescent protein (RFP), ''smart'' probes, and near-infrared fluorescent (NIRF) probes, and bioluminescence imaging, which utilizes systems such as Firefly luciferase/D-Luciferin or Renilla luciferase/coelenterazine. Emission of light from fluorescent markers requires external light excitation, while bioluminescent systems generate light de novo when the appropriate substrates/cofactors are made available. In both cases, light emitted from either system can be detected with a thermoelectrically cooled charge-couple device camera (CCD) since they emit light in the visible light range (400 nm to 700 nm) to near-infrared range (~800 nm). Cooled to −120 to −150°C, these cameras can detect weakly luminescent sources within a light-tight chamber. Being exquisitely sensitive to light, these desktop camera systems allow for quantitative analysis of the data. The image shown above the Fluorescence Imaging schematic is representative of the one obtained from a glioma model which expresses RFP (image used with permission from Anticancer, Inc.). The method of imaging bioluminescent sources of living subjects with a CCD camera is relatively straightforward: the animal is anesthetized, subsequently injected with the substrate, and immediately placed in the light-tight chamber. A light photographic image of the animal is obtained, which is followed by a bioluminescence image captured by the cooled CCD camera positioned above the subject within the confines of the dark chamber. The 2 images are subsequently superimposed on one another by a computer, and relative location of luciferase activity is inferred from the composite image. An adjacent color scale confers relative concentration of luciferase activity. Sample image above the Bioluminescence Imaging schematic is a typical image obtained with this technology (image courtesy of Bhaumik and Gambhir, Ref. 15. In this specific example, the image was obtained after intravenous injection of coelenterazine into a mouse containing intraperitoneal *Renilla* luciferase-expressing tumor cells. Significant bioluminescence is detected from the region of the xenograft. See the color insert for the color version of this figure.

within the cell. In contrast, FDG clears from cells or tissues that lack the ability to transport or phosphorylate FDG. The positron emitting moiety of FDG, in this example, ^{18}F, decays by emitting a positron from its nucleus. This positron eventually collides with a nearby electron, resulting in an annihilation event where two 511,000 eV photons in the form of gamma rays are emitted ~180° apart. The 2 emitted photons travel extracorporeally and are detected nearly simultaneously as they interact with the PET ''camera''—a ring array of detectors (composed of scintillation crystals and photomultiplier tubes) surrounding the subject (Fig. 2). Detection of a single annihilation event results in the ''activation'' of detectors opposing one another, which is recorded as a ''coincident event'', thus defining a set of coincident lines (25). The recording of multiple detector pair combinations yields a large number of these coincident lines. Sophisticated mathematical analyses of the coincident lines, which include filtered back projection and attenuation correction, yield the location of cell populations or tissues that have accumulated FDG. Tomographic images of relative probe concentration can be reconstructed in the sagittal, coronal, and transverse imaging planes. Quantitative information obtained from the images is, in turn, related to the underlying biochemical process.

Radiolabeling molecules is not just limited to ^{18}F. A collection of positron-emitting isotopes is available for use, which includes the more commonly utilized isotopes ^{15}O, ^{13}N, ^{11}C, and ^{18}F, and the less commonly used ^{14}O, ^{64}Cu, ^{62}Cu, ^{124}I, ^{76}Br, ^{82}Rb, and ^{68}Ga. Most of these isotopes are created in a cyclotron, a device used to accelerate charged particles to create the relatively short-lived positron-emitting isotopes (for example, ^{18}F, the half-life of which is 110 minutes) (26). Automated synthesizers can then couple the isotope to a molecule of interest to produce the molecular probe (tracer). Given the relatively short half-life of positron emitters, the process of producing isotopically labeled molecules has to be performed with great efficiency and in relatively close proximity to the hospital, clinic, or animal research facility. In this regard, a modest number of PET radiopharmacies are available worldwide, producing PET tracers on a daily basis.

Clinical PET scanners have been around for several decades and, in recent years, PET cameras for small animals have been developed for the purpose of developing molecular imaging assays in small rodents prior to their application in humans. The spatial resolution of most *clinical* PET scanners is ~$(6–8)^3$ mm^3 with the more recent scanners achieving ~$(3)^3$ mm^3 capabilites. By comparison small animal PET scanners (microPET) have a resolution of ~$(2)^3$ mm^3 with newer generation scanners attaining ~$(1)^3$ mm^3 (27,28). When compared to other modalities, the sensitivity of PET is relatively high—on the order of 10^{-11} to 10^{-12} moles/liter (MRI's intrinsic sensitivity is ~10^{-4} to 10^{-5} M) (29). Furthermore, the location depth of the tracer of interest does not affect sensitivity. In contrast, the imaging of many optical imaging probes is significantly affected by tissue depth. Under appropriate conditions, the smallest cluster of cells that can be visualized by a clinical PET scanner is 10^6 to 10^9 in number. Thus, radiotracer imaging techniques afford the detailed location(s), magnitude, and persistence of probes or tracers for in vivo use in animals and humans.

C. Basic Principles of the Gamma Camera and SPECT imaging

Imaging with a gamma camera is similar to PET, but the radiolabel emits gamma rays instead of positrons. A variety of radiolabels, each emitting at characteristic photon energies, can be attached to molecules including ^{111}In (171, 245 keV), ^{125}I (27–35 keV), ^{131}I (364 keV), and ^{99m}Tc (140 keV). Once introduced into the body, detection of these radiolabeled probes is performed with a gamma camera, a scintillation detector consisting of collimator, a sodium iodide crystal, and a set of photomutliplier tubes (Fig. 3). Upon decay, these radionuclides emit a gamma ray at their characteristic energies in different directions. Some of the gamma rays will scatter or lose energy and others may never interact with the camera. Since the gamma camera is situated only on 1 side of the subject, only rays directed toward the camera will be ''captured''. Furthermore, only those gamma rays that arise parallel to the collimator will be detected since the collimator will absorb scattered gamma rays. Those rays that successfully reach the crystal will be converted into photons of light. In turn, the photomultiplier tubes convert the light into an electrical signal that is proportional to the incidental gamma ray. Gamma rays, which arrive at the detector lower than the expected characteristic energy, are thought to be the result of scattering and summarily rejected from the analysis. Since gamma cameras acquire data in a single plane, the resultant images are a 2-dimensional representation of a 3-dimensional subject (referred to as ''planar imaging'').

While more affordable and accessible than PET, the limitations of gamma camera imaging are obvious: 1) diminished sensitivity, since many decay events are either rejected or never captured, 2) decreased signal-to-noise since overlapping foci of activity are not delineated, and 3) lower spatial resolution. Alleviating some of these problems is single photon emissions computed tomography (SPECT), which acquires volumetric data by rotating the gamma camera around the subject and/or using multidetector systems (30) (Fig. 3). As with microPET, small animal SPECT devices (microSPECT) have been created to study the use of gamma-emitting radiolabeled reporter probes in animal models of cancer and gene therapy. These instruments have resolutions on the order of 1 mm^3. Thus, advantages of the SPECT systems are that they generally allow for better spatial resolution. Another advantage of SPECT is that 2 radioisotopes of different energies can be imaged simultaneously, allowing for the concurrent study of 2 distinctly radiolabeled molecules (1 radiolabeled with ^{99m}Tc and another radiolabeled with ^{125}I). With PET imaging, such simultaneous imaging is not possible since all positron emitting events are 511keV.

A disadvantage for SPECT, however, is that it is an order of magnitude lower in sensitivity than PET. To accommodate for the loss of sensitivity, more radiolabeled probe and, thus,

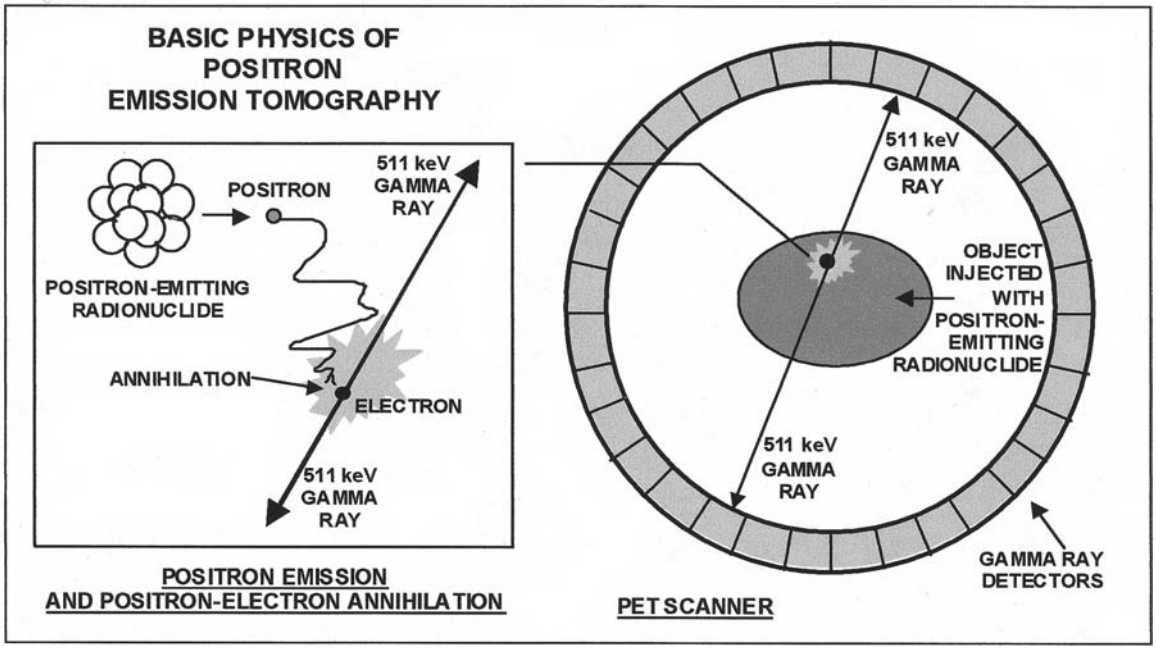

Figure 2 Basic Principles of Positron Emissions Tomography (PET) Imaging Biologically active molecules such as glucose, peptides, and proteins can be radiolabeled with positron-emitting radioisotopes. This radiolabeled molecule is referred to as a probe or tracer. The positron-emitting isotope decays by emitting a positron from its nucleus. This positron eventually collides with a nearby electron, resulting in an annihilation event where 2 511,000 eV photons in the form of gamma rays are emitted ~180° apart. The 2 emitted photons travel extracorporeally and are detected nearly simultaneously as they interact with a ring of detectors (composed of scintillation crystals and photomultiplier tubes) surrounding the subject. Detection of a single annihilation event results in the ''activation'' of detectors opposing one another, which is recorded as a ''coincident event.'' The recording of multiple detector pair combinations yields a large number of these coincident lines. Sophisticated mathematical analyses of the coincident lines, which include filtered back projection and attenuation correction, yield the location of cell populations or tissues that contain the molecule labeled with the positron emitter. Tomographic images of relative probe concentration can be reconstructed in the conventional sagittal, coronal, and transverse imaging planes or, actually, in any arbitrary plane. The resultant image depicts the distribution and concentration of the radiolabeled tracer. Sensitivity of PET is in the range of 10^{-11} to 10^{-12} moles/liter and is independent of the location depth of the tracer of interest. It is also important to note that all positron-emitting radioisotopes produce 2 gamma rays of the same energy, so if 2 molecular probes—each with a different positron-emitting isotope—are injected simultaneously, there is no way for the PET camera to distinguish between the 2 molecular probes. Therefore, to perform studies that look at 2 or more distinct molecular events (e.g., suicide gene therapy and imaging apoptosis, cardiac gene therapy, and perfusion ^{13}N ammonia imaging, etc.), one has to inject molecular probes separately, which allows decay of the isotope.

higher levels of radioactivity have to be injected into the subject to maximize signal to noise.

D. Basic Principles of Magnetic Resonance Imaging

With regards to the electromagnetic spectrum, MRI works with longer wavelengths (lower energies) than those used by radionuclide or optical techniques. A comprehensive review of MRI is beyond the scope of this chapter; there are several excellent reviews and books available (31,32). A very brief, simplistic description follows here: Spinning charged nuclei generate a magnetic field, and MR imaging depends on these charged atomic nuclei, which contain an odd number of protons. Those nuclei that contain an odd number of protons always have an unpaired proton, which gives the atom a net magnetic field or ''magnetic dipole moment'' (MDM). In contrast, atoms, which have an even number of protons, have a net magnetic field of zero. Thus, the nucleus of hydrogen (^{1}H), which possesses a single unpaired proton as its nucleus and is present in abundance (in the form of H_2O and $-CH_2-$) in cells and tissues, is primarily used for MR imaging.

The majority of available MR scanners are outfitted with a superconducting magnet in the shape of a long hollow tube. The magnet creates an ''external magnetic field'' (B_0) and, within the hollow center of the tube-shaped magnet, the mag-

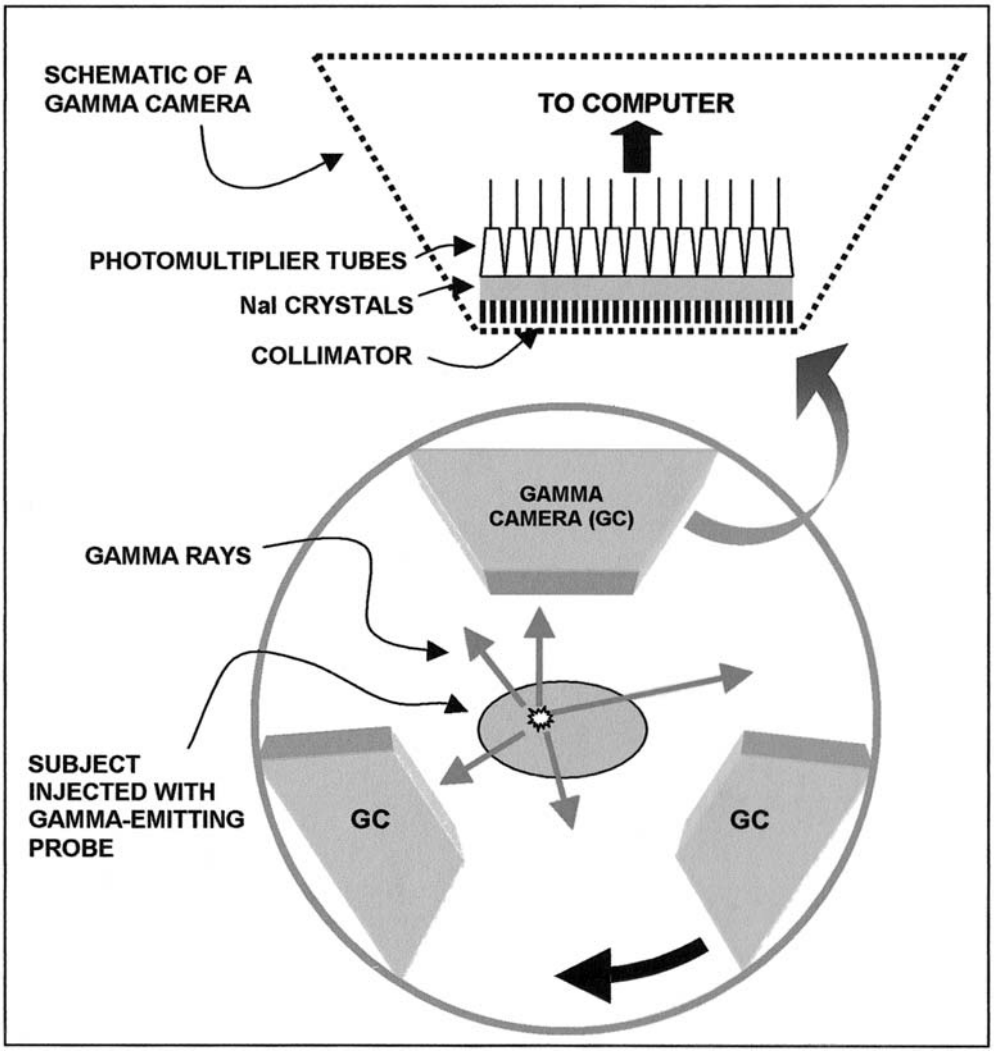

Figure 3 Basic Principles of Gamma Camera/SPECT Imaging Imaging with a gamma camera is similar to PET, but the radiolabel emits gamma rays instead of positrons. A variety of radioisotopes, each emitting at characteristic photon energies, can be attached to a variety of molecules. Examples of isotopes include ^{111}In (171, 245 keV), ^{125}I (27–35 keV), ^{131}I (364 keV), and ^{99m}Tc (140 keV). Once introduced into the body, detection of these radiolabled probes is performed with a gamma camera, a scintillation detector consisting of collimator, a sodium iodide crystal and a set of photomultiplier tubes. Upon decay, these radionuclides emit a gamma ray at their characteristic energies in different directions. Some of the gamma rays will scatter or lose energy and others may never interact with the camera. Since the gamma camera is situated only on 1 side of the subject, only rays directed toward the camera will potentially be ''captured''. Furthermore, only those gamma rays that arise parallel to collimator will be detected since scattered gamma rays will be absorbed by the collimator. Those rays that successfully reach the crystal and are stopped by it will be converted into photons of light. In turn, the photomultiplier tubes convert the light into an electrical signal that is proportional to the incidental gamma ray. Gamma rays that arrive at the detector lower than the expected characteristic energy are thought to be the result of scattering and summarily rejected from the analysis. Since gamma cameras acquire data in a single plane, the resultant images are a 2-dimensional representation of a 3-dimensional subject (referred to as ''planar imaging''). Single photon emission computed tomography (SPECT) acquires volumetric data by rotating a gamma camera around the subject and/or using multidetector systems (shown above). (From Ref. 30.) See color insert for color version of this figure.

netic field is nearly homogeneous and parallel to the long axis of the tube. When a subject is placed within the hollow confines of the magnet, the MDMs of the hydrogen atoms align themselves with the main magnetic field B_0- much like the way iron filings behave when placed in the vicinity of a magnet.

Once equilibrium has been established in the external magnetic field, B_0, a magnetic pulse (an electromagnetic wave), otherwise known as radiofrequency (RF) pulse, is introduced perpendicular to B_0. It is called an RF pulse because the frequency (and energy) of the pulse is in the radiofrequency range of the electromagnetic spectrum (3–100 MHz). This causes the hydrogen nuclei to transiently orient their MDM parallel to the new magnetic field (perpendicular to B_0). After the RF pulse, they realign (relax) their MDMs to the main magnetic field B_0, and, in the process give off energy in the form of radiofrequency waves that can be detected by receiver coil, which typically surrounds the subject. To reiterate, both a RF pulse and magnetic field are used to perturb the underlying subject, with an RF wave being generated in the process that is used to produce an image. Also, the time it takes hydrogen nuclei to relax to equilibrium (or a fraction thereof) can be measured. The rate at which a hydrogen nucleus relaxes is dependent upon the nature of its parent molecule such as a freely mobile water proton vs. the rigidly attached proton of hydrocarbon backbone of a fatty acid. Water protons, which randomly tumble in aqueous solution, take longer to regain equilibrium with the main magnetic field, B_0, than those protons associated with much larger, more fixed molecules. The measurements of relaxation rates can be converted into a value, which translates into image pixel value, with each pixel representing a small, representative, unit volume of the subject (voxel). On a certain MR imaging protocol called a ''T1-weighted'' sequence, a voxel composed mostly of fatty (hydrocarbons) protons will have a high (bright) signal since the rate of relaxation is rapid. Compare this to the voxel that contains a large number of water protons: this voxel will have a low (dark) signal on T1-weighted MR imaging since the rate of relaxation is much longer. Each MR image is 256 × 256 or 512 × 512 pixels, each a representative slice through the subject (Fig. 4).

Certain exogenous or endogenous atoms/molecules, like Gadolinium (paramagnetic) and iron/hemosiderin (superparamagnetic), respectively, can influence the local magnetic field by their powerful magnetic properties, significantly alter the rate of relaxation of the protons and therefore generate contrast in the image. Paramagnetic substances have unpaired electrons. They become magnetized in the presence of an external magnetic field and contribute to an increase in the effective magnetic field. Examples include the rare-earth element, gadolinium (Gd) (7 unpaired electrons), deoxyhemoglobin (4 unpaired electrons), and methemoglobin (5 unpaired electrons). Hemosiderin, an end-stage by-product of hemorrhage, has more than 10,000 unpaired electrons and, thus, belongs to *superparamagnetic* group of substances. The magnetic susceptibility of superparamagnetic substances is 100 to 1000 times stronger than paramagnetic substances. Both super- and paramagnetic substances help localize reporter gene expression during MRI (see also Fig. 10). As we will see in a later section, the properties of paramagnetic and superparamagnetic compounds are exploited for MR-based reporter systems.

MRI techniques offer phenomenal spatial resolution (voxel resolutions of ~10 μm^3 in vitro and ~50 μm^3 in small animals) but are several orders of magnitude less sensitive than optical and radionuclide-based techniques. Sensitivity of MRI is on the order of 10^{-3} M while PET imaging is 10^{-12} M, and thus, substantially more MRI probe has to be injected into the living subject in order to provide sufficient contrast (33).

E. Computed Tomography

Computed tomography (CT) deals in the X-ray range of the electromagnetic spectrum. In CT, the subject is placed in the center of a ring of detectors. A rotating, focused X-ray source emits radiation that penetrates the subject and reaches a set of detectors on the other side of the patient. The amount of X-ray reaching the detector depends upon the amount absorbed by the patient and is inversely proportional to the density of tissues encountered as it passes through the patient. The amount of radiation reaching a detector is given a value, and, through a complex set of back calculations, tomographic images in the transverse plane can be constructed.

While this modality is not currently utilized to monitor gene expression, efforts are under way. Rather, CT's main role in human gene therapy will grow significantly as it currently serves as an anatomical adjunct to PET imaging. Both CT and PET data sets can be coregistered, and because of its superior spatial resolution, CT gives PET information more specificity. Clinical CT scanners have recently achieved spatial resolutions under 1 mm. Small animal CT scanners (microCT) have attained resolutions of 50 μm (34). Already showing its prowess in oncology, PET/CT clinical machines as well as small animal machines should help to show gene expression coupled to anatomy. More details about PET/CT can be found elsewhere (23).

III. REPORTER GENES AND REPORTER PROBES

A. Optical Reporter Systems

There are essentially 4 different types of optical reporter genes currently in use for studies in living animals. These genes either encode a) a protein that contains a chromophore (a short, internal peptide segment that contributes to the protein's fluorescent capabilities following obligatory posttranslational modifications), which fluoresces when externally irradiated (fluorescence imaging), b) an enzyme that can convert an exogenously added, optically quiescent substrate into a fluorescent complex, or an enzyme that changes the conformation of a substrate such that it fluoresces a different color (fluorescence imaging), c) luciferases that generate light when presented with the appropriate substrate (bioluminescence imaging), or d) fusion proteins that couple transgene products with

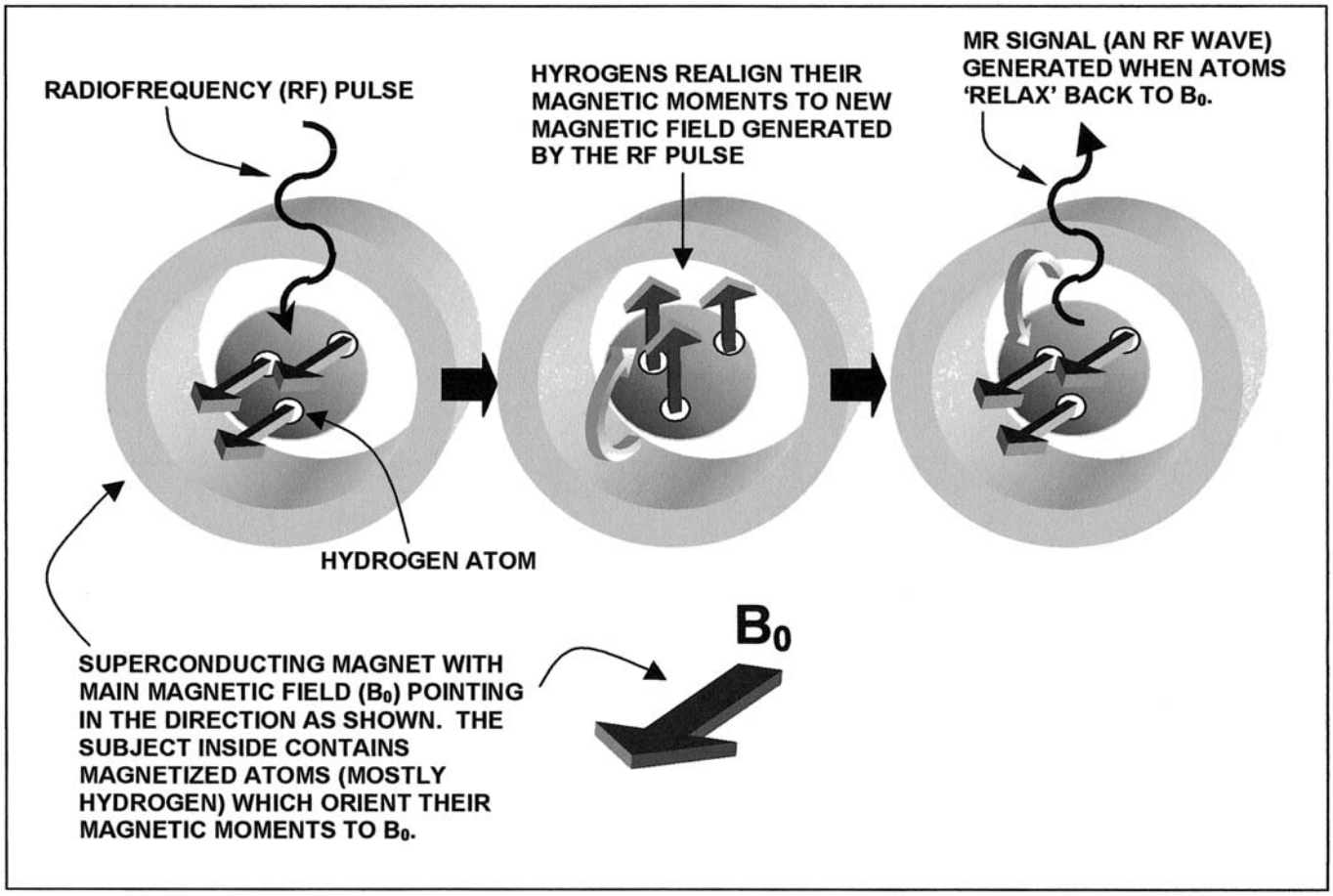

Figure 4 Basic Principles of Magnetic Resonance Imaging (**MRI**) In MRI, subjects are placed in a strong, external, magnetic field, B_0, produced by a hollow, cylindrical magnet. The B_0 field is nearly uniform and points parallel to the long axis of the magnet. Imaging with MRI is dependent upon atomic nuclei with an odd number of protons, such as hydrogen (^1H). Such atoms have their own net magnetic field [a.k.a., magnetic dipole moment (MDM)] and their moments align accordingly when placed in this external magnetic field. Once equilibrium has been achieved between the subject and the magnet, energy can be added to the system in the form of a radiofrequency (RF) pulse. In most cases, this pulse, which generates its own magnetic field, can change the alignment of the hydrogen atoms such that their moments are now perpendicular to B_0. Once the RF pulse is ''turned off'', the hydrogen atoms realign or ''relax'' to B_0 and give up energy in the form of RF waves during the relaxation period. Receivers located in the magnet capture this RF wave. One of the calculations made from the captured information is the rate at which the hydrogen atoms relax to equilibrium. Image construction and image contrast are possible with MRI because hydrogen atoms associated with macromolecules like fat and proteins have a significantly different relaxation rate than the hydrogen atoms of bulk water. The measurements of relaxation rates can be converted into a value, which translates into image pixel value, with each pixel representing a small, representative, unit volume of the subject (voxel). On a certain MR imaging protocol called a ''T1-weighted'' sequence, a voxel composed mostly of fatty (hydrocarbons) protons will have a high (bright) signal since the rate of relaxation is rapid. In contrast to voxels that contain a large number of water protons, this voxel will have a low (dark) signal on T1-weighted MR imaging since the rate of relaxation is much longer. See color insert for color version of this figure.

fluorescence or bioluminescence optical reporters with a peptide linker (discussed in greater detail in a later section). When compared with other imaging modalities, fluorescence and bioluminescence imaging techniques hold tremendous potential for study of small living animals because of their relative affordability, relative ease of use, high assay sensitivity, and low requirement for specialized support personnel. In contrast to the radionuclide-based techniques described earlier, all optical reporter gene/probe systems are forms of indirect imaging. The measured light emissions generated from these re-

porter systems may or may not correlate to the amount of therapeutic gene product present. Briefly reviewed below, extensive work in the past several years has been dedicated to mutating existing or cloning new fluorescent or bioluminescence genes that are more compatible for use in living subjects.

Mammalian tissues pose a number of obstacles for the propagation of light and, thus, are a challenge for the evaluation of fluorescence or bioluminescence-based reporter genes in living subjects. Fluorescent-based techniques, in particular, face additional challenges since both the light used to excite the reporter probe and the light emitted from the probe are subject to absorption, scatter, and other optical tribulations. More specifically, the excitation light is not only limited by its ability to penetrate nontransparent tissue, but also contributes to background autofluorescence (from hemoglobin and cytochromes) especially when excitation wavelengths are in the blue and green portions of the spectrum, which is generally the case when green fluorescent proteins (GFP) [and its close relatives such as blue fluorescent proteins (BFP)] are used as reporter genes (21). As a result, fluorescence imaging has relatively poor signal-to-noise when compared to bioluminescent techniques.

Light emitted from either fluorescence-or bioluminescence-based techniques is subject to tissue absorption and scattering. In mammalian tissues, the blameworthy structures responsible for light absorption are predominantly molecules of hemoglobin, which absorb wavelength emissions of 400–600 nm. To a lesser extent, melanin and other pigmented macromolecules also contribute to light absorption, and, thus, experiments using white-furred or hairless subjects are preferred when using optical reporter systems. Light absorption by water molecules is also another important factor, but not until wavelengths approach >900 nm range. Light scatter is another important confounding factor in the detection of low-level photon emissions; the interfaces at the surface membranes of cells and organelles are largely the cause for this occurrence (21).

Yet, despite these impediments, recent advances in the fields of optics and sensor technology make it possible to detect relatively low emission events with great sensitivity and generate remarkable images (35,36). Furthermore, optical reporters are being developed to operate with longer wavelengths; that is, away from the blue-green part of the spectrum and towards the red (a.k.a. ''red-shifted,'' between 600–900 nm) so as to maximize transmission, minimize absorption, and minimize background autofluoresence. Additionally, the fluorescence efficiency of each of the fluorescent proteins is being optimized through site-directed mutagenesis. The intrinsic physical and chemical properties of the mutants are altered. Characteristics such as protein stability, extinction coefficients, fluorescence quantum yield, tendency to dimerize or form multimers, requirement for oxygen, efficiency of fluorochrome formation, and susceptibility to photoisomerization and photobleaching can be modulated through mutagenesis, and, thus, the amount and rate of light photons emitted from such structures can be optimized (37).

For example, wild-type *Aequorea* GFP, a 238 amino acid polypeptide (27–30 kD) specifically isolated from the Pacific jellyfish (*Aequoea victoria*), is a highly fluorescent molecule with excitation peaks at 395 nm (largest peak) and 475 nm (38). GFP's emission peak is 509 nm, which is in the lower green portion of the visible spectrum. On the skin surface, GFP expression can be easily visualized. However, for optimal visualization of GFP expression in deeper structures, such as the brain and pancreas, skin or skull windows need to be created by surgical incision (39). Additionally, GFP fluorescence is not immediate and only detectable at about 7 h after injection of recombinant adenovirus carrying GFP (vAd-CMV-GFP). The rate-limiting step in GFP ''maturation'' appears to be a necessary oxidation step in chromophore formation (40).

Extensive work has been dedicated to creating GFP mutants, since wild-type GFP possesses a few factors compromising its use in living subjects. The list of available GFP mutants, each having their own idiosyncrasies, is quite lengthy. An excellent review is available (37). One of the more thoroughly studied mutants S65T, also known as enhanced GFP (EGFP), has some impressive advantages. Ser65, one of the amino acids of the chromophore, is replaced by Thr in this mutant. The wild-type 395 nm excitation peak is suppressed, and the 475 nm peak is enhanced 5- to 6-fold in amplitude (6-fold increase in brightness), the peak is shifted to 489–490 nm (40). It is 4-fold faster during the rate-limiting oxidation step, not subject to photoisomerization, and exhibits very slow photobleaching (41). Examples of the use of EGFP in living subjects is shown in Fig. 5.

GFP mutants come in all kinds of colors: blue, cyan, yellow, and green. However, the maximum emission peak attained by the GFPs is 529 nm. Recently cloned red fluorescent protein (dRFP), a 28-kDa protein responsible for red coloration seen in the coral *Discosoma*, has broken the 529 nm barrier, and excitation and emission maxima are at 558 nm and 583 nm, respectively (42). The relatively high extinction coefficient and fluorescence quantum yield indicate that the brightness of the mature, well-folded protein is comparable to any other fluorescent protein. Furthermore, a commercially available mutant, DsRed, is resistant to photobleaching and has been further red-shifted to 602 nm, which reduces the tissue absorption of the emitted light photon. Unfortunately, there are some significant limitations to the use of dRFP. It is an obligate tetramer and is quite slow in its maturation—it takes days for it to mature from green to red. Mutations attempted thus far have not been able to alleviate these problems (42). Figure 1 contains a fluorescent image example of an RFP-expressing glioma in a mouse model.

Two recently developed ''smart'' fluorescent probes have been developed that are significantly red-shifted—so much that they work in the near-infrared portion of the electromagnetic spectrum (excitation wavelength 673 nm; emissions wavelength of 689 nm), making them ideal fluorescent agents for use in intact organisms. Although they are being exploited to measure endogenous protein levels, they are worth mentioning here since they have the potential to be activated by exoge-

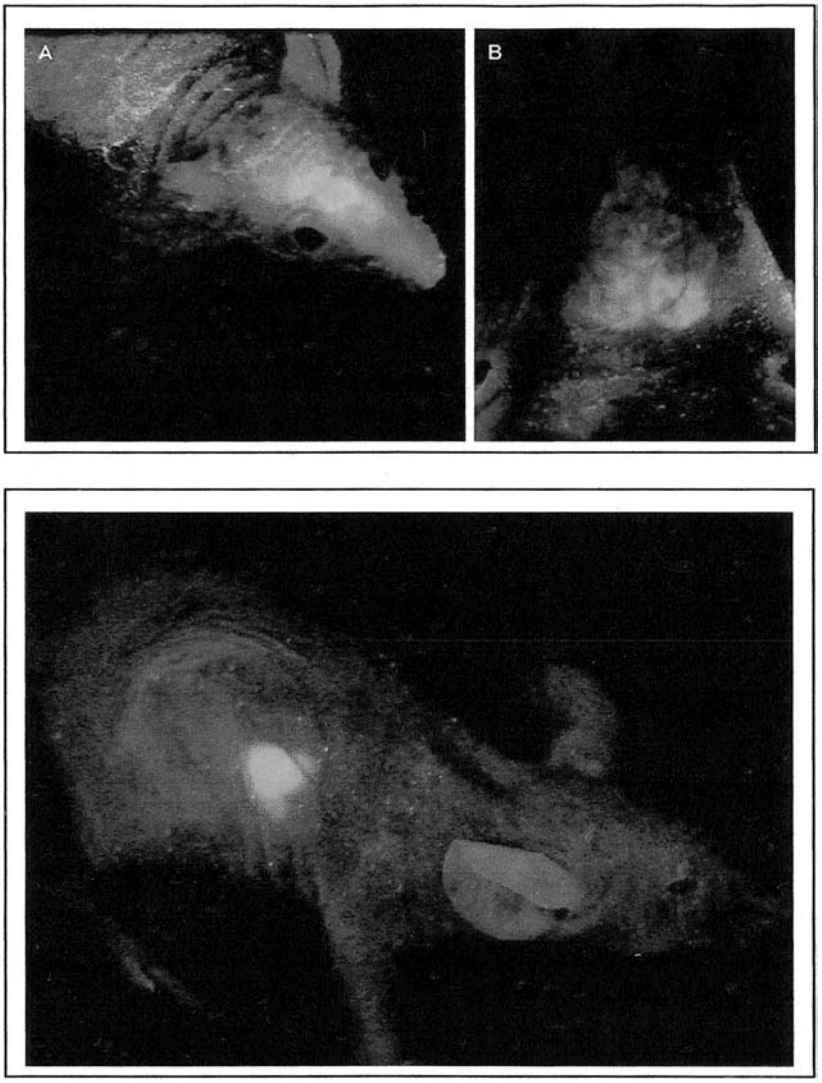

Figure 5 Optical (Fluorescence) Imaging of Transgene Expression The adenoviral (vAd) vector AdCMV5GFPAE1/AE3 [vAd-green fluorescent protein (GFP)] (Courtesy of Quantum, Montreal, Canada) constitutively expresses an enhanced GFP (eGFP), which is driven by a CMV promoter. The vector was delivered to the brain after an upper midline scalp incision and creation of a parietal skull window. Twenty mL containing 8×10^{10} plaque-forming units (pfu)/mL vAd-GFP per mouse was injected into the brain. Twenty-four hours later, fluorescence imaging of the entire animal (lower magnification) was carried out in a light box illuminated by blue light fiber optics, which provided the external excitation wavelength, and was imaged using a cooled color charge-coupled device camera. Emitted fluorescence was collected through a long-pass filter GG475 on a 3-chip thermoelectrically cooled, color CCD camera. Images of 1024×724 pixels are captured directly on a personal computer or continuously through video output on a high-resolution video recorder. Images are subsequently processed for contrast and brightness and analyzed with imaging software. Higher magnification images (not shown here) can be accomplished by using a fluorescence stereomicroscope equipped with a 50 W mercury lamp. In this scenario, selective excitation of GFP is produced through a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence are captured and processed as described above. Images A and B demonstrate GFP transgene expression following adenoviral delivery to the brain. Image C demonstrates Ad-CMV-GFP delivery to the liver via portal vein cannulation. See color insert for color version of this figure. (Images courtesy of Anticancer, Inc., Ref. 18.)

nously delivered genes and to be used to monitor gene therapy (19). This new breed of fluorescent probe is dependent upon the close proximity of multiple near-infrared fluorochromes (NIRF), Cy5.5, when bound to a synthetic graft copolymer consisting of a cleavable backbone [partially methoxy poly(-ethylene glycol)-modified poly-L-lysine] (43). When placed in close proximity, a pair of these fluorochromes will ''quench'' each other and, therefore, not be detectable. Upon enzymatic cleavage of the backbone with a protease that has lysine-lysine specificity, the fluorochromes are spatially dissociated and will begin to fluoresce.

Because tumor progression and angiogenesis necessarily elaborate certain proteases, these clever or ''smart'' biocompatible autoquenched near-infrared fluorescent probes can be used to detect tumors that are known to upregulate certain proteases. Cathepsins B and H are tumor proteases that have lysine-lysine specificity and have been shown to activate this fluorescent probe in tumor xenografts. Other known tumor-enhanced proteases, Cathepsin D and matrix metalloproteinase-2 (MMP-2), which are dependent on other specific peptide sequences for their action, can also activate this probe if the NIR fluorochromes are attached to the backbone via Cathepsin D-sensitive or MMP-2-sensitive sequences; these enzyme-specific probes have been demonstrated in living subjects (19,44).

Bioluminescent optical systems are increasingly being used in the study of living subjects because of their inherently low background (no excitation irradiation needed that would otherwise cause background autofluorescence). The most commonly used bioluminescence reporter gene is the Firefly luciferase gene, *Fluc*, which encodes a 550 amino acid, 61-kDa monomeric protein (FL) derived from *Photinus pyralis*, the North American firefly (36). Photon emission is achieved through oxidation of its native substrate, D-Luciferin [D-(−)-2-(6′-hydroxy-2′-benzothiazolyl) thiazone-4-carboxylic acid] into oxyluciferin in a reaction that requires ATP, Mg^{2+}, and O_2. The reaction produces a broad spectral emission that peaks at 560 nm. A number of modifications to the gene since its discovery has facilitated its use in mammalian tissues, which include amino acid substitutions that red-shift the emissions peak above 600 nm, optimized mammalian codon language, removal of a peroxisome targeting site for increased expression levels, and cytoplasmic localization (21,45). Once produced, luciferase does not require post translational processing for enzymatic activity, and it can immediately function as a genetic reporter. Examples of the use adenoviral-mediated firefly luciferase gene delivery are given in Figs. 6 and 7. Figure 6 is an example of cardiac gene therapy and Fig. 7 is an example of how tissue-specific transgene expression can be achieved using tissue-specific promoters in living subjects.

A second bioluminescence system utilizing the *Renilla* luciferase gene (*Rluc*) that encodes a 36-kDa monomeric protein (RL), has recently been tested in small rodents (15). Its peak emission displays a blue-green bioluminescence at 480 nm when *Renilla* luciferase interacts with its substrate, coelenterazine. In comparison to FL, RL does not need cofactors or ATP to oxidize its substrate, and therefore it will be less taxing to the cell in which it is expressed. It also has much more rapid kinetics in terms of light production so that it can potentially be used simultaneously with FL through the injection of both substrates and multiple time-point imaging (15).

Comparing the technical and practical aspects of fluorescent and bioluminescence systems reveals substantial differences, which are briefly mentioned here. Fluorescence imaging benefits greatly from relatively high photon yield from its proteins and dyes so that, in some cases, living subjects can be imaged with conventional photographic equipment rather than the more expensive, cooled CCD cameras. In comparison, the photon yield of bioluminescence systems is significantly lower, and imaging of living subjects always requires a cooled CCD camera. Fluorescence imaging can potentially be used to image simultaneous signals of different colors, whereas multiplexing different and simultaneous signals in bioluminescence imaging is difficult, owing to the differential enzyme kinetics and time-to-peak photon flux among varying luciferases.

On the other hand, bioluminescence imaging has certain advantages over the fluorescence systems. For example, autofluorescence, a source of noise in the image, is significantly less in bioluminescence for reasons discussed earlier. Also, bioluminescence reporters can be utilized as soon as they are synthesized. In contrast, the fluorescent proteins usually have a requisite period, some substantially longer than others, of posttranslational processing and maturation prior to their function as a fluorescent reporter (37). In some cases, where temporal resolution is an important issue in the monitoring of gene therapy, the use of fluorescence techniques may be limiting in this regard. Bioluminescence proteins also take some finite time to mature, but anecdotal evidence suggests that it is not as long as the fluorescent proteins; future studies are certain to address this issue.

Imaging with bioluminescence techniques permits rapid, repetitive, or prolonged imaging periods so long as the required substrates and cofactors are available. The physical phenomena of photobleaching, a property seen in many fluorescent proteins where the fluorochrome is permanently extinguished after light excitation or ultraviolet light exposure, makes imaging difficult if prolonged excitation periods or rapid repetitive imaging is needed; repeat imaging can only take place once fresh fluorescent protein has been generated. Photobleaching is not an issue in bioluminescence imaging. Having stated the above, no formal comparison has yet been made between fluorescence and bioluminescence using the same animal model, fusion reporter/individual reporters, and the same CCD camera. These studies should help to better define which is the more ideal reporter.

B. Radionuclide-Imaging Reporter Systems

Currently, PET or Gamma/SPECT reporter genes encode either a receptor, enzyme, or ion pump, which bind a radiolabeled ligand, interact with a radiolabeled substrate, or facilitate intracellular translocation of ionic radioisotopes, respectively. The reporter gene product is designed to sequester the

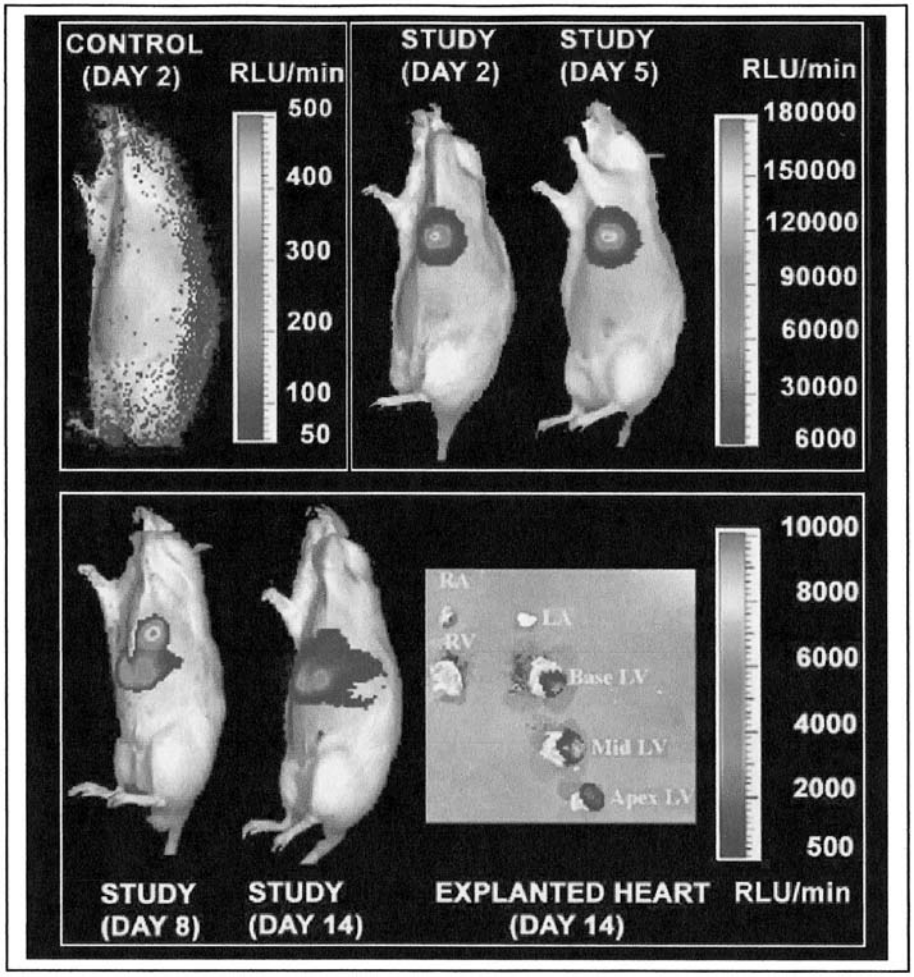

Figure 6 Optical (Bioluminescence) Imaging of Cardiac Reporter Gene Expression Replication-defective adenovirus carrying firefly luciferase (*Fluc*) driven by a constitutive cytomegalovirus (CMV) promoter (Ad-CMV-*Fluc*, 1×10^9 pfu) was injected directly into the myocardium (anterolateral wall) of a rat. Images obtained 2, 5, 8, and 14 days later from a cooled CCD camera demonstrate significant cardiac emissions from firefly luciferase activity ($P<0.05$ vs. control). By day 8, luciferase activity is seen in the liver, which is probably from spillover of adenoviral vector into the systemic circulation and subsequent hepatic transfection of the virus via coxsackie-adenovirus receptors on hepatocytes. On day 14, the heart of the same rat was explanted after whole-body imaging was performed and sliced into 3 sections (bottom right). Firefly luciferase activity is localized at the anterolateral wall of the left ventricle along the site of virus injection. Control rats, which received an intracardiac injection of Ad-CMV-HSV1-sr39*tk* (1×10^9 pfu), demonstrate no significant firefly luciferase activity 2 days after the injection (upper left). Please note the bioluminescent scales are different for control rat, study rat days 2 to 5, and study rat days 8 to 14 to account for the wide range of cardiac firefly luciferase activity observed. Scales are a quantitative indicator of light photons detected [relative light units (RLU)/minute (min)]. RA indicates right atrium; RV, right ventricle; and LV, left ventricle. See color insert for color version of this figure. (Images reproduced with permission, Ref. 116.)

probe intracellularly and/or on the surface of cells expressing these genes. Ideally, those cells lacking the transgene will be unable to trap the reporter probe. The amount of probe or tracer used is typically in the nanogram range and does not lead to any pharmacological effect (46). In contrast, bioluminescence strategies, NIRF and MRI, require mass amounts of probe (usually μg–mg) and, as a result, may produce a pharmacological effect.

Radionuclide imaging has significant advantages in that it permits quantitative and repetitive imaging in the same subject over time (47–50). Perhaps the main drawback of radionuclide-based techniques is that unsequestered probe circulates

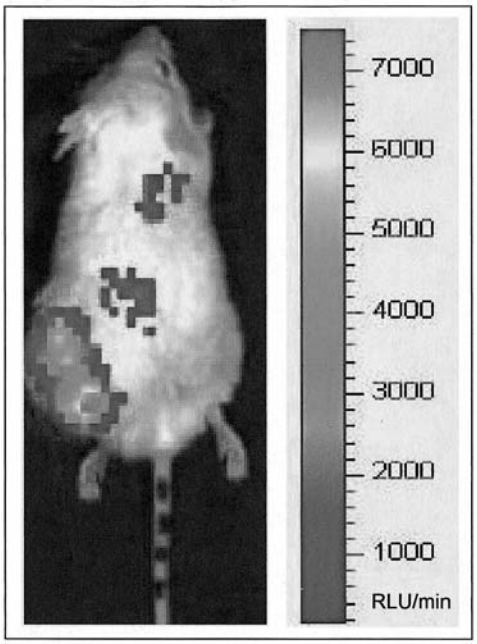

Figure 7 Optical (Bioluminescence) Imaging of Targeted Transgene Expression Using a Tissue-Specific Promoter One way to target gene therapy is through the use of tissue-specific promoters. However, most tissue-specific promoters yield low levels of transcription. In this example, certain key regulatory elements of the promoter and enhancer of prostate-specific antigen (PSA) have been multimerized to yield a construct, PSE-BC, which is 20-fold more active than the native PSA promoter/enhancer. Following incorporation into an adenovirus vector (AdPSE-BC-luc) and subsequent intratumoral injection into a human prostate cancer xenograft model (LAPC series), firefly luciferase expression can be seen in the main tumor xenograft (left flank) as well as other extratumoral sites, such as the back and chest, in this male SCID mice 21 days after vector delivery. Detailed histological analysis of the xenograft and extratumoral sites demonstrates that firefly luciferase expression is restricted to the prostate tumor and prostate metastases, respectively. The metastases, in this case, are located in the spine and lung. By comparison, CCD imaging and histological analysis of xenografts injected with AdCMV-luc show markedly diminished expression of firefly luciferase in the xenograft and increased nonspecific expression in the liver at 21 days postinjection (figure not shown). Results from this study indicate that tissue-specific transgene expression is possible and that CCD imaging can be used to track firefly luciferase-marked tumor cells. Scale indicates the number of photons detected (RLU/min). See color insert for color version of this figure. (Images reproduced with permission, Ref. 117.)

in the enterohepatic system or is excreted in the kidneys. As a result, probe can collect in the gut, kidneys, urinary bladder, or gall bladder, making it difficult to specifically evaluate these organs. Discussed below are 2 fundamentally different approaches to radionuclide-based reporter imaging: direct and indirect imaging.

1. Direct Radionuclide Imaging: HSV1-TK, Mutant HSV1-TK, Sodium Iodide Symporter, and Cytosine Deaminase

One of the major advantages of using a radionuclide technique is that it allows the opportunity to directly image delivered therapeutic genes (i.e., ''direct imaging''). In direct imaging, there is no need to couple the therapeutic gene with an additional reporter gene since reporter probes already exist for the therapeutic gene. The herpes simplex virus type 1 thymidine kinase gene (HSV1-*tk*) is an exemplary model of direct imaging.

Mammalian thymidine kinases phosphorylate thymidine for its normal incorporation into DNA during replication. The HSV1 thymidine kinase gene (HSV1-*tk*), on the other hand, is normally employed as a suicide gene product for the therapy of cancer. The gene product, with its broad substrate specificity, is able to phosphorylate acycloguanosine, guanosine, and thymidine derivatives and subsequently trap these substrates intracellularly much more efficiently than endogenous thymidine kinase. Thus, HSV1-*tk* acts as a suicide gene when delivered with a prodrug (such as acyclovir, ganciclovir, and penciclovir) since high concentrations of phosphorylated nucleoside analogs cause premature chain termination by their inhibition DNA polymerase, which leads to cell death. Transfected cell populations particularly affected by this type of therapy are those that have a high mitotic index. When radiolabeled acycloguanosines (e.g., 9-[(3-[^{18}F] fluoro-1-hydroxy-2-propoxy)methyl]guanine ([^{18}F]FHPG); 9-(4-[^{18}F] fluoro-3-hydroxymethylbutyl)guanine ([^{18}F]FHBG)), guanosines (e.g., fluorogangciclovir ([^{18}F]FPGV); fluoropenciclovir ([^{18}F]FPCV)), and thymidine derivatives (2′-[^{124}I] fluoro-2′deoxy-1-β-D-arabinofuranosyl-5-iodouracil ([^{124}I]FIAU)) are used in nonpharmacological (trace) doses, they can serve as PET reporter probes. Similarly, [^{131}I]FIAU and [$^{123/125}$I]FIAU can be used as gamma camera or SPECT reporter probes. The details of the synthesis and kinetics of these and other similar agents have been reviewed (51–56).

Endogenous thymidine kinase, HSV1-*tk*, and a mutated form of HSV1-*tk*, HSV1-sr39*tk*, each demonstrate different substrate specificity that can be exploited for therapeutic and imaging purposes. For example, endogenous thymidine kinase demonstrates narrow substrate specificity and cannot efficiently phosphorylate radiolabeled prodrugs. While endogenous thymidine kinase can phosphorylate the prodrugs/probes to a minor degree, nontransfected cells cannot accumulate significant amounts of the radiolabeled prodrugs. Instead, these radiolabeled prodrugs preferentially localize to cells, tissues, or tumors that express the HSV1-*tk* gene.

Comparison of the HSV1-*tk* probes demonstrates that [^{124}I]FIAU displays favorable pharmacokinetics (greater sen-

sitivity, better contrast, less background noise) and, thus, the best imaging potential (57). [^{124}I]FIAU, in particular, may be advantageous over the other tracers in some cases because the half-life of ^{124}I is 4.2 days, allowing for longer systemic clearance. On the other hand, this longer half-life may also prove problematic in cases when frequent repeat imaging is needed since a minimum 10 to 12 days between imaging sessions is needed to allow clearance of prior probe administration. Further testing has shown that [^{18}F]FHPG and [^{18}F]FHBG are inferior agents for HSV1-TK. This variability seen amongst HSV1-TK reporter probes is in part due to differences in biological half-life, stability, substrate competition, degree of nonspecific binding, specific retention, rates of cellular transport, method of transfer (viral-mediated vs. stable transfection), and routes of clearance (58).

Mutated versions of the suicide gene, HSV1-*tk*, have been created to enhance its killing potential by increasing its ability to phosphorylate prodrugs (59). From a library of site-directed mutants, it has been determined that the product of mutant HSV1-sr39*tk* suicide gene is more adept at phosphorylating ganciclovir and less efficient in phosphorylating endogenous thymidine when compared to wild-type HSV1-TK. In light of this, mutant kinase has been exploited as a reporter gene since radiolabeled reporter probes already exist and are identical to those used for the wild-type HSV1-TK. As expected, HSV1-sr39TK efficiently phosphorylates [^{18}F]FGCV, [^{18}F]FPCV, [^{18}F]FHPG, and [^{18}F]FHBG, with [^{18}F]FHBG as the most effective substrate for the mutant thymidine kinase. The ability to trap the reporter probe is improved by at least a factor of 2.0–3.0 in mutant TK-expressing tumor xenografts when compared with wild-type TK. Significantly improved uptake of the reporter probe is seen in the liver following systemic delivery of a recombinant adenoviral vector carrying the mutated transgene, resulting in enhanced sensitivity for imaging this transgene in vivo when compared to wild-type HSV1-*tk* (60). An example of the use of HSV1-sr39*tk* as a potential reporter gene for cardiac gene therapy is provided (Fig. 8). As an aside, an example of an optical reporter for use in cardiac gene therapy (using a nearly identical adenoviral vector and the firefly luciferase reporter gene) is provided for side-by-side comparison to better demonstrate the spatial advantages of microPET imaging (Fig. 8).

Another therapeutic transgene that lends itself to direct imaging in living subjects is the sodium/iodide symporter (NIS) gene (Fig. 9). NIS is an intrinsic membrane protein that is responsible for translocating and concentrating iodide within thyroid follicular cells. In this normal, physiological situation, iodide is eventually used to make the thyroid hormones (61). Recent cloning of the symporter has permitted the investigation of its role as a suicide gene. Because the symporter can concentrate high intracellular levels of iodide (including radioiodide, ^{131}I), targeted cells expressing the symporter can be killed in this form of targeted radiotherapy; by the accumulation of radioiodide, it is estimated that a dose of up to 50,000 cGy of ionizing radiation can be achieved in targeted cancer cells (62)! The lethal effects of the NIS have been cleverly demonstrated in vitro, in a variety of cell lines including melanoma, colon cancer, and ovarian carcinoma, and in a murine model of a transfected melanoma xenograft following intraperitoneal injection of ^{131}I (63). Also, tissue-specific expression of NIS transgene is possible by fusing a prostate-specific antigen (PSA) promoter fragment with the NIS gene—the result being tissue-specific expression of NIS transgene and subsequent accelerated death of an androgen-dependent prostatic carcinoma xenograft after injection with a therapeutic dose of radioiodine (64).

Imaging of the NIS transgene is relatively straightforward since the therapeutic agent, ^{131}I, can also be used as an imaging agent with a gamma camera or SPECT. Similarly, ^{123}I or ^{125}I, both of which are commercially available, can also be used as reporter probes. A significant advantage of this reporter system is that the reporter probe is relatively simple to produce and commercially available. Specialized radiochemistry, such as that required for HSV1-*tk* reporter probes, is not needed here. Another significant advantage of this system is that this reporter system can readily be imaged with PET using ^{124}I as the positron-emitting reporter probe, which already is available (65); the very same living subject carrying the NIS transgene can be imaged with either SPECT or PET depending upon the choice of reporter probe. The main confounding issue with using this reporter system, on the other hand, is that radioiodine will not only localize to target cells, but will also accumulate in normal tissues such as the thyroid, salivary glands, breast, and stomach, all of which express physiological levels of endogenous NIS. As it currently stands, it is also not clear if the NIS approach is as sensitive as the HSV1-*tk*/sr39*tk* approaches.

The cytosine deaminase (CD) transgene, another suicide gene, encodes an enzyme that converts 5-fluorocytosine to the toxic 5-fluorouracil (5-FU). Efforts to image this transgene, which have been performed with radiolabeled fluorocytosine as a probe, have largely been hampered by suboptimal pharmacokinetics: poor tracer uptake and poor retention of the toxic metabolite (66). Extended periods of up to 48 h are required to see differential accumulation of tracer between transfected and control cells. While targeted delivery of CD remains a viable method of suicide gene therapy (67), alternative substrates for CD, which are rapidly transported, deaminated, and trapped intracellularly, will have to be developed in order to use this transgene as a reporter gene.

2. Indirect Radionuclide Imaging: D2R, Mutant D2R, and Somatostatin-2 Receptor

In cases where a reporter probe does not already exist for a delivered transgene, a number of strategies exist for indirect transgene imaging. Indirect imaging involves the simultaneous coexpression of the therapeutic gene and reporter gene with both driven by the same or identical promoter. Because reporter gene expression can directly correlate with transgene expression, such approaches have the potential to give us valuable information on the quantity and localization of transgene expression (68). It should be noted, however, that it is not a given that they directly correlate; one can hope that they do but each approach/application has to be tested. Further details

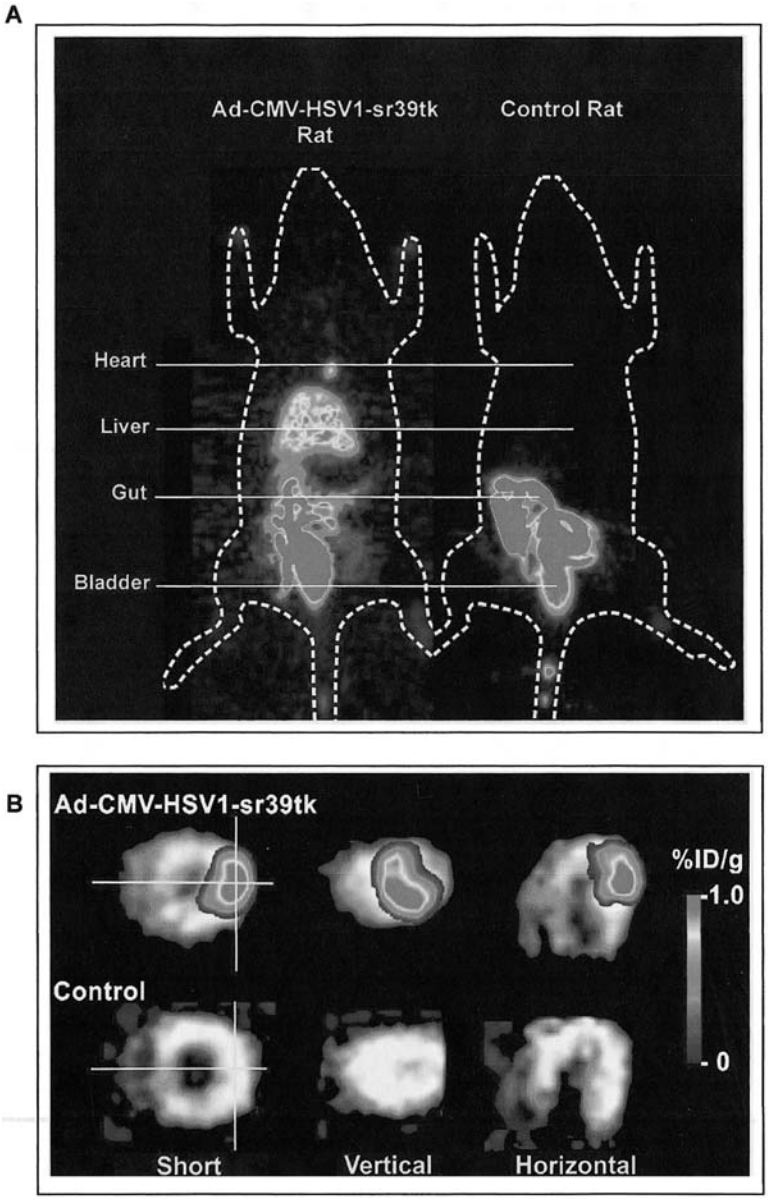

Figure 8 Micropet and Optical (Bioluminescence) Imaging of Cardiac Reporter Gene Delivery (A) Imaging cardiac gene expression using adenoviral-mediated mutant thymidine kinase (HSV1-sr39*tk*) as PET reporter gene and [^{18}F]FHBG as PET reporter probe. Trapping of tracer occurs only in cells expressing the reporter gene. At day 4, whole-body microPET image of a rat shows focal cardiac [^{18}F]FHBG activity at the site of intramyocardial Ad-CMV-HSV1-sr39*tk* injection. Liver [^{18}F]FHBG activity is also seen because of systemic adenoviral leakage with transduction of hepatocytes. Control rat injected with Ad-CMV-*Fluc* shows no [^{18}F]FHBG activity in either cardiac of hepatic regions. Radiolabeled probe is always ''visible'' with radionuclide imaging regardless of whether it has localized to its target or not. As a result, radionuclide-based images will exhibit a certain degree of nonspecific tracer localization since the ''unbound'' reporter probe is metabolized through either the enterohepatic or urinary system or both. In this example, nonspecific reporter probe activity and gut and bladder activities are seen for both study and control rats because of route of [^{18}F]FHBG clearance. (B) Tomographic views of cardiac microPET images. The [^{13}N]NH_3 (gray scale) images of perfusion are superimposed on [^{18}F]FHBG images (color scale), demonstrating HSV1-sr39*tk* reporter gene expression. [^{18}F]FHBG activity is seen in the anterolateral wall for experimental rat compared with background signal in control rat. Perpendicular lines represent the axis for vertical and horizontal cuts. Color scale is expressed as % ID/g. (C) Comparison of typical images obtained with PET (left) and optical imaging (right). The optical method is more sensitive (at limited depths), easier to perform, and demonstrates minimal background noise. With PET, we can see that the transgene was delivered to the anterolateral aspect of the left ventricle. Such spatial resolution is not afforded by in vivo optical imaging at this time. See color insert for color version of this figure. (Images reproduced with permission from Refs. 116,118.)

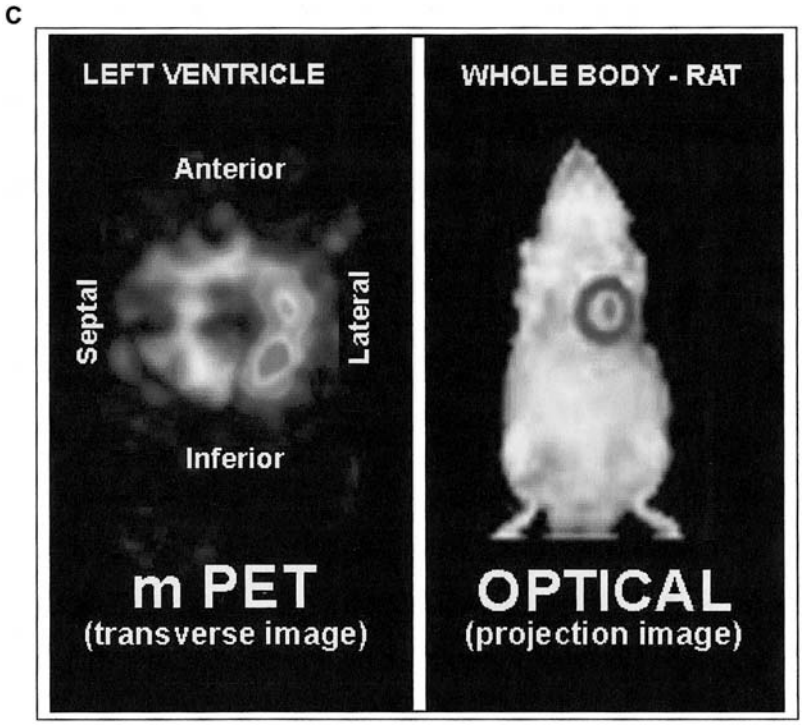

Figure 8 Continued.

regarding such indirect approaches are provided in a later section.

In radionuclide imaging, one way to perform indirect imaging is to couple the therapeutic gene to a reporter gene that encodes a receptor that can bind, and, therefore, trap radiolabeled ligands. Following transfection, targeted cells may express the therapeutic gene product and receptor in proportional amounts. Subsequently, the ectopically expressed receptor will either localize to the cell membrane and/or remain intracellular. Following exposure to radiolabeled ligand probe, cells expressing the receptor will specifically bind the probe, resulting in a complex that can be detected by PET/SPECT/gamma camera imaging. The intensity of activity on the PET image is directly proportional to the number of these receptor-ligand complexes, and, therefore, correlated to the amount of therapeutic gene expressed. Ideally, those cells not producing the receptor will be devoid of tracer signal.

An example of a receptor-based reporter gene is the dopamine type 2 receptor reporter gene (D2R) (49). D2R is normally an endogenous, cell-surface receptor predominantly expressed in the striatum. When activated, it causes a G-protein-coupled reduction of cyclic adenosine monophosphate (cAMP) via its inhibition of adenylate cyclase. When D2R is used as a reporter gene, a radiolabeled D2R antagonist, spiperone [3-(2′ [^{18}F]fluoroethyl)spiperone ([^{18}F]FESP)], serves as the receptor's ligand and accumulates intracellularly and on the cell surface of D2R-expressing tissue. Radiolabeled spiperone, originally used to monitor levels of endogenous levels of striatal D2 receptors in vivo, binds to D2R with high affinity and is able to cross the blood-brain barrier. To overcome potential deleterious effects of *ectopic* D2R activation by circulating *endogenous* ligands, mutant D2R reporter genes, D2R80A and D2R194A, have been created that are disengaged from downstream transduction events while maintaining a high binding affinity for ligand probe (48).

A few advantages of the D2R-based reporter system are worth commenting. [^{18}F]FESP's ability to cross the BBB and cell membranes favors its use in the central nervous system relative to other reporter systems such as the HSV1-*tk* (mutant or not) reporter systems since [^{18}F]FHBG is not as efficient in crossing this important barrier. Furthermore, [^{18}F]FESP has a relatively easier time of localizing to target, which is a cell-surface and intracellular receptor. In contrast, [^{18}F]FHBG has to cross the cell membrane in order to interact with the target enzyme and is, therefore, subject to transport kinetics. Also, D2R, an endogenous protein, is not immunogenic compared to HSV1-TK and therefore probably more appropriate for repeated imaging during longitudinal studies. Interestingly, despite these relative advantages of the D2R/[^{18}F]FESP system, equivalent sensitivities are reported between the D2R/[^{18}F]FESP and mutant HSV1-*tk*/[^{18}F]FHBG PET reporter gene imaging systems in the liver (~20% ID/g in the liver when used with adenoviral delivery systems carrying constitutive CMV-based expression of the reporter gene). One must

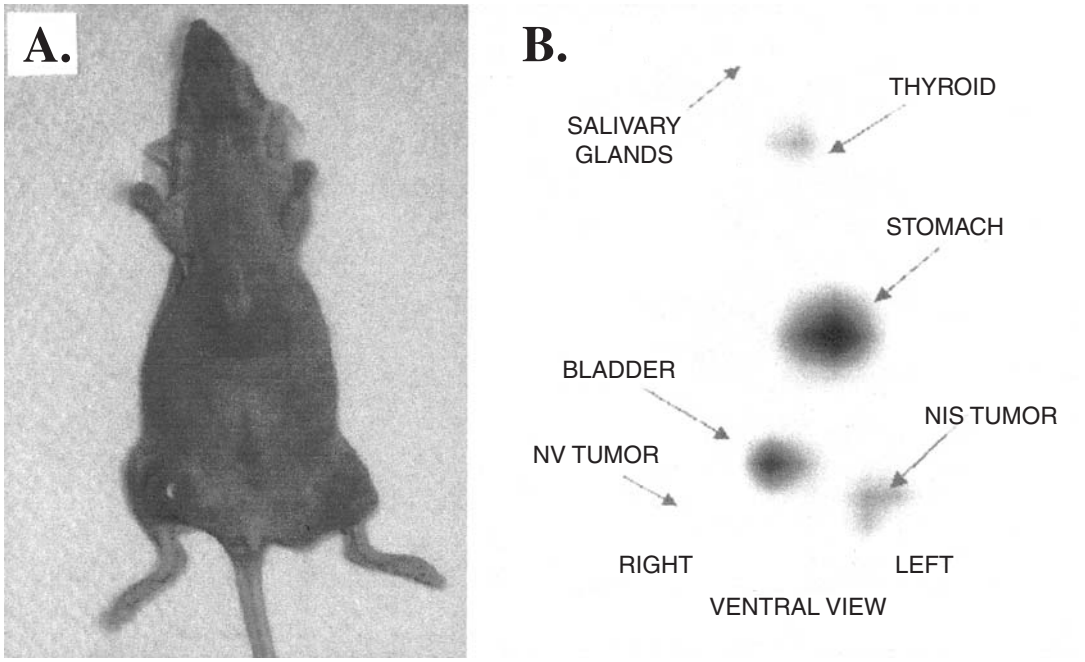

Figure 9 Gamma Camera Imaging of the Sodium Iodide Symporter Transgene Recently cloned rat and human sodium iodide symporter genes (rNIS and hNIS, respectively) are increasingly showing their potential as a novel suicide gene therapy for a variety of cancer models. Once transduced to a cancer cell line, tumor, or target organ via recombinant viral transfection or liposomal-mediated techniques, the expressed membrane proteins facilitate the active intracellular transport of iodide (I^-) into targeted cells. Exogenously administered radiotracers ^{123}I, ^{125}I, or ^{99m}Tc-pertechnetate results in intracellular accumulation of these tracers in cells that are transduced with this symporter. The distribution of these tracers can be imaged with a gamma camera or SPECT and, therefore, be used as a means of localizing cells that have been transduced with NIS. Similarly, targeted brachytherapy can be performed by the administration of ^{131}I. (A) A retroviral vector carrying the rNIS gene was used to transduce A375 human melanoma cell line. Transduced (NIS) and nontransduced (NV) tumor xenografts were subcutaneously implanted into the left and right flank of the photographed mouse, respectively. (B) By 30 days, the tumor had reached approximately 10 mm in diameter. An intraperitoneal dose of ^{131}I was administered, and a gamma camera image was obtained after a 1-h incubation period. rNIS-transduced xenograft (left flank) demonstrates radioiodide uptake while nontransduced tumor (right flank) does not. The thyroid, stomach, and, to a lesser extent, the salivary glands endogenously express sodium iodide symporters and, thus, normal, physiological radioiodide uptake is seen in these organs. (Images reproduced with permission from Ref. 63.)

also note that endogenous D2R expression in the striatum will produce background noise and, therefore, [^{18}F]FESP imaging will only be useful outside the striatum.

Another receptor-based reporter system takes advantage of the somatostatin membrane receptors (SSTR), which also belong to the family of G-protein-coupled receptors. Under normal conditions, the interaction between SSTR and its ligand, somatostatin (SS), is known to have a variety of biological effects including a role in vasoconstriction, immunomodulation, and an inhibitory effect on endocrine and exocrine secretory functions; more recently, SSTR-activated signal transduction pathways have been implicated in the induction of apoptosis and inhibition of cell growth (69) (70). SSTRs have also been found in normal and hyperplastic human endothelium where it is felt that the SSTR exerts negative effects on angiogenesis. Five different somatostatin receptor subtypes have been described thus far (SSTR1 through SSTR5), and their respective genes have been cloned (71).

Historically, the development of this reporter gene centered around probes that were already in existence and were being used clinically to identify diseased states characterized by upregulated SSTR receptors. Radiolabeled somatostatin analogs, for example, have been clinically used to identify a number of primary human cancers and their metastases where elevated levels of somatostatin receptors are seen. Neuroendocrine tumors (including carcinoid, islet cell tumors, small-cell lung cancers, pheocromocytomas, gastrinoma, paragangliomas, and medullary thyroid cancers), pituitary gland tumors as well as sarcomas, meningiomas, low-grade astroctomas, lymphomas, some breast cancers, and metastatic prostate cancers are known to express high levels of SSTR, particularly SSTR2. In fact, the combination of high SSTR receptor den-

sity seen in some tumors and the antiproliferative, antiangiogenic, and antisecretory effects of SS analogs form the premise for somatostatin analog therapy for cancer patients (72,73).

The favorable binding kinetics of SS-SSTR are the basis for use of the *sstr2* gene as reporter gene. Currently, [^{111}In]-DTPA-D-Phe^{1}-octreotide (Octreoscan) and [^{99m}Tc]-depreotide (Neotect) are radiolabeled somatostatin analogs that have been in routine clinical use for the past several years for the imaging of SSTR-positive tumors using gamma cameras or SPECT, i.e., SSTR scintigraphy (74,75). These agents have also been useful in the imaging of non-neoplastic conditions that are associated with SSTR upregulation, such as a variety of autoimmune and granulomatous diseases.

The potential of using *sstr2* as a reporter gene has been realized. Fig. 10 demonstrates adenoviral delivery via intratumoral injection of human *sstr2* gene into a tumor xenograft of a mouse. The transfected tumor is subsequently detected with a gamma camera following an intravenous injection of [^{99m}Tc]P2045, a somatostatin analog (76). For *sstr2* to serve as a true reporter gene, however, it will have to be uncoupled from signal transduction since it may have undesireable effects in targeted and surrounding cells in its current state.

On a side note, *sstr2* is being tried as a ''double-edged'' suicide transgene for cancer therapy. Not only does SSTR primarily mediate antimetabolic effects as described earlier, but it can also be utilized for its ability to internalize and retain SS analogs. Efforts are being made to deliver cytotoxic agents to targeted cells by using the receptor as a courier of toxic SS analogs, such as [^{90}Y]-DOTA-D-Phe^{1}-Tyr^{3}-octreotide ([^{90}Y]-SMT 487). ^{90}Y exerts its lethal effects by local irradiation via emitted β particle. When SSTR-null tumor xenografts are injected with recombinant adenovirus encoding the SSTR2 receptor, they become susceptible to systemically administered [^{90}Y]-SMT 487 (77). This version of targeted radiotherapy is able to significantly reduce quadrupling times of the xenograft. When the *sstr2* transgene is used in this manner it can be directly imaged using Octreoscan.

Indirect imaging can also be accomplished by coupling the therapeutic gene to a reporter gene that encodes an enzyme which converts freely dispersible radiolabeled substrate probes into sequestered products (78). For example, previously described HSV1-*tk* or HSV1-sr39*tk* can be used as a reporter gene by using [^{124}I]FIAU or [^{18}F]FHBG at tracer levels (subpharmacological, nontherapeutic dose). In this manner, the HSV1-*tk* or mutant counterpart can be utilized strictly as a reporter gene.

C. MRI Reporter Genes

Imaging transgene expression using MRI depends on reporter genes that encode receptors or enzymes which specifically interact with probes which are attached or chemically modified to accommodate paramagnetic or supraparamagnetic substances. Once localized to their targets, these probes alter the local magnetic field, which changes the relaxivity of nearby protons, and, concomitantly, effects a change in the radiofrequency signal detected by the receiver. At least two MRI reporter systems have been proven in animal studies: engineered transferrin receptor (ETR)-dependent reporter systems and the *lacZ*-EgadMe system.

Iron (Fe) is a superparamagnetic ion that can cause significant changes of the local magnetic field, which can be detected by MRI if supraphysiological concentrations can be achieved. Transferrin (Tf), an iron-binding protein, and its cell-surface receptor (Tf-R), a receptor ubiquitously present on most cell types, mediate normal cellular iron metabolism and regulation. Normal intracellular stores of iron are dependent upon internalization kinetics of this receptor-ligand complex. By removing the 3′ untranslated region (UTR) regulatory sequence from the Tf-R gene and mRNA destabilization motifs in the 3′ untranslated region, engineered transferrin receptor (ETR) has been created to be constitutively overexpressed and liberated from feedback regulatory control (79,80). As expected, ETR-transfected cells accumulate approximately 500% more probe (holo-Tf) than control cells. To further augment the difference between transfected and control cells, the reporter probe itself has been modified to possess even greater magnetic susceptibility characteristics. It involves the synthesis of a 3 nm monocrystalline iron oxide nanoparticle (MION) that is surrounded by a layer of low molecular-weight dextran to which holotransferrin is covalently bound (Tf-MION). On the average, each MION particle contains approximately 2000 superparamagnetic Fe atoms (compared with the 2 Fe atoms present in a single molecule of the paramagnetic chelate, holotransferrin). As expected, T2-weighted gradient echo MR imaging (1.5T; imaging time 3–7 min per sequence; voxel resolution 300 × 300 × 700 μm) reveals significantly lower signal intensity in ETR^{+} tumor xenografts than control following intravenous administration of Tf-MION (79). Recently, a second superparamagnetic reporter probe, a dextran cross-linked iron-oxide (CLIO) superparamagnetic particle conjugated to trasnferrin (Tf-CLIO) has also been effective in identifying ETR+ tissues with MR imaging (81).

The other MR imaging method shown to be compatible with living subjects relies on an enzymatic amplification strategy to monitor gene expression. As mentioned before, gadolinium (Gd) is a rare-earth element with the largest number of unpaired electrons. With 7 unpaired electrons, Gd is a strong paramagnetic substance, collectively affecting the spins of water protons immediately surrounding it. The increased signal (for T1-weighted sequences) seen in Gd-enhanced MR imaging is afforded by the increased relaxation rate of intimately associated water protons surrounding a Gd atom. Gadolinium's ability to affect the relaxation rate of protons varies inversely to the distance between the paramagnetic ion and water protons.

Based on these principles, a reporter probe, (1-(2-(β-galactopyranosyloxy)propyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane)gadolinium(III) (EgadMe), has been cleverly formed by ''encasing'' Gd in a water-proof package—an artificial barrier or cage designed to keep water molecules at bay so as not to be affected by the Gd's magnetic personality (Fig. 11A) (82). In this configuration, water has

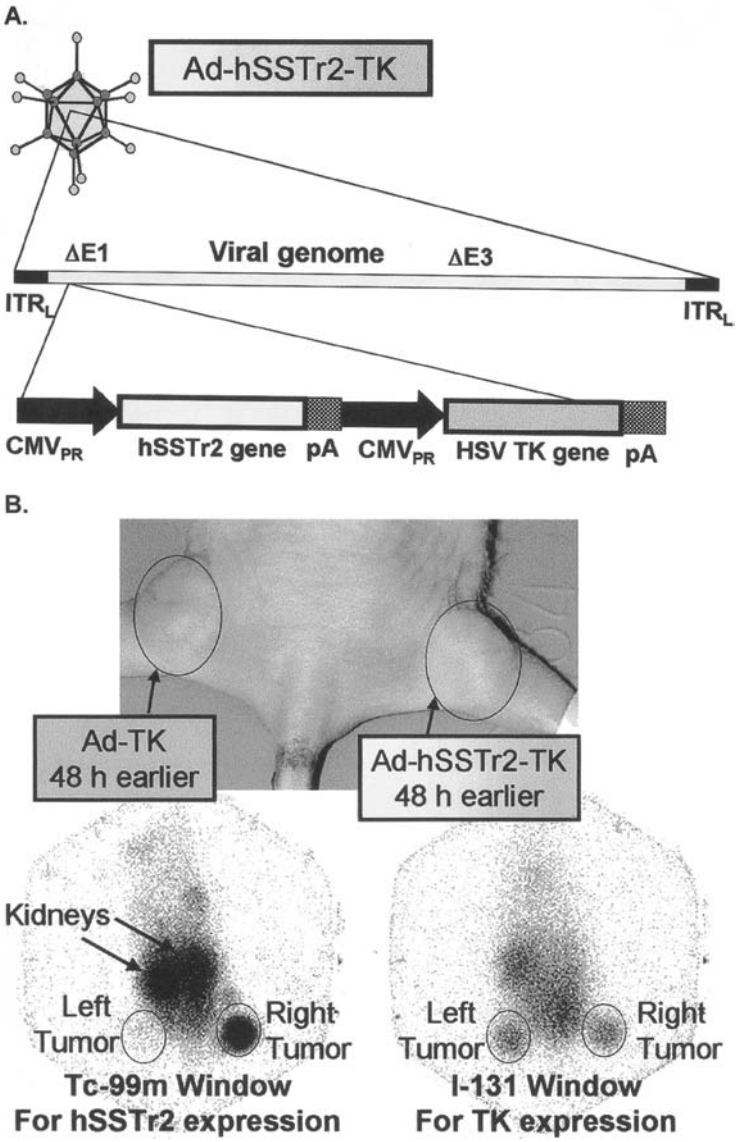

Figure 10 Gamma Camera Imaging of a Dual Promoter Construct (A) To measure target (therapeutic) gene expression, several strategies are employed to ''link'' the therapeutic gene with a reporter gene on a single vector. In one strategy, indirect measurements of a target gene expression can be made by a downstream reporter gene with both genes driven by separate and identical promoters (i.e., the 'dual-promoter' construct). Replication-incompetent adenovirus encoding human type 2 somatostatin receptor (*hsstr2*) and the herpes simplex thymidine kinase (TK) enzyme (Ad-CMV-*hsstr2*-CMV-*tk*) is an example of this construct. Both transgenes are driven by the cytomegalovirus promoter element. (B) Human nonsmall cell lung cancer tumor xenografts were subcutaneously implanted in the right and left flank of a mouse. The left tumor was injected with Ad-CMV-tk and the right tumor was injected with Ad-CMV-*hsstr2*-CMV-*tk*. Forty-eight h later, the mouse was simultaneously injected (IV) with both ^{99m}Tc-P2045, which is a somatostatin receptor peptide ligand, to detect expression of *hsstr2* and radioiodinated ^{131}I-2′-deoxy-2′-fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU) to detect TK expression. Imaging was performed with an Anger gamma camera 5 h after injection of the radiotracers. The gamma camera can be 'tuned' to select for gamma rays that fall within a defined range. It is this property of the gamma camera that can discriminate among the activity of different radioisotopes such as ^{99m}Tc (140 keV) and ^{131}I (364 keV). Thus, 2 images can be obtained from the same animal by changing the window settings in the gamma camera—one for ^{99m}Tc (for *hsstr2* expression, lower left image) and the other for ^{131}I (for TK expression, lower right image). The right flank xenograft, which was injected with the dual promoter construct (Ad-CMV-*hsstr2*-CMV-*tk*), demonstrates uptake of both radiotracers, while the left flank xenograft (injected with Ad-CMV-*tk*) demonstrates [^{131}I]FIAU uptake only. These finding support the feasibility of the dual promoter approach for tracking transgene delivery. (Images reproduced with permission from Ref. 76.)

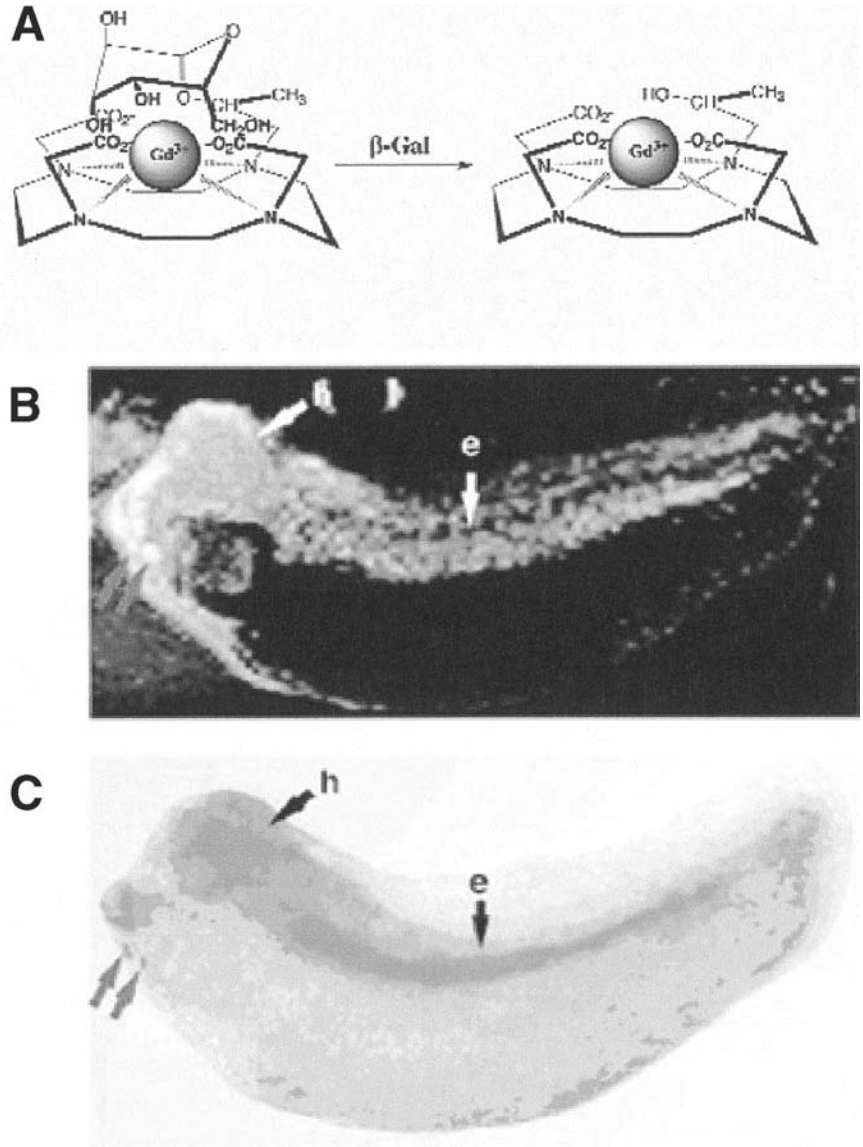

Figure 11 Magnetic Resonance Imaging (MRI) of β-galactosidase-activated MRI Reporter Probe (EgadME) One can obtain image contrast in MRI by using paramagnetic substances that change the local magnetic field and, thereby, increase the relaxation rate of nearby water protons. Gadolinium (Gd) is an example of a paramagnetic substance, and a relatively high local concentration of this agent translates into enhanced brightness as seen on T1-weighted images. (A) A reporter probe, EgadMe, has been formed by 'encasing' Gd in a molecular casing—an artificial barrier designed to keep water molecules at bay so as not to be affected by the Gd's magnetic effects. In this configuration, water has no access to the paramagnetic ion, and therefore, this probe is 'silent' on MR imaging. Part of the physical barrier is composed of a sugar, a galactopyranose cap, which has been attached to the cage by a β-galactosidase-cleavable linker. If *lacZ* is used as a reporter gene, subsequent enzyme cleavage releases the cap and allows water access to the gadolinium ion, thus, 'activating' this novel MR contrast agent. (B) EgadMe permits MRI detection of *lacZ* gene expression. Linearized plasmid cDNA encoding *lacZ* is injected into 1 of the cells of the 2-cell stage *Xenopus laevis* embryo. EgadMe is injected into both cells of the 2-cell stage. Subsequent enzyme expression is on 1 side of the animal since the 2 cells represent the future right and left sides of the animal. MR imaging of the embryos has been obtained at approximately the 100,000-cell stage using a 11.7 T magnet. As expected, β-galactosidase activity is seen in one half of the animal depicted as areas of high signal intensity within the endoderm (e) and head (h). (C) Light microscopic images of the same embryo fixed and stained with X-gal. Areas of X-gal staining follow regions of high signal intensity on MR image. (Images reproduced with permission from Ref. 82.)

no access to the paramagnetic ion and is therefore this probe is ''silent'' on MR imaging. As cleverly designed, part of the physical barrier is composed of a sugar—a galactopyranose cap, which has been attached to the cage by a β-galactosidase-cleavable linker. If *lacZ* is used as a reporter gene, subsequent enzyme cleavage releases the cap and allows water access to the gadolinium ion, thus, ''activating'' this novel MR contrast agent. β-galactosidase activity, following introduction of linearized plasmid cDNA encoding *lacZ* into a specific subset of cells in a *Xenopus laevis* embryo, has been imaged after an intracellular injection of EgadMe (Fig. 11B,C).

In its current state, EgadMe has difficulty crossing cell membranes and, as a result has to be directly injected intracellularly to maximize detection in vivo. Furthermore, relatively slow kinetics of cleavage for this agent is perhaps suboptimal for imaging gene expression (82). Regardless, these relatively recent developments will be refined, and they indicate great potential for MR imaging of transgene expression.

D. Magnetic Resonance Spectroscopy (MRS) Reporter Genes

Certain metabolites produced physiologically from endogenous enzymes or uniquely from exogenous enzymes have unique chemical signatures that can be detected using MRS (a.k.a., nuclear magnetic resonance (NMR) spectroscopy). These enzymes can be overexpressed in target tissues and can be used as MRS reporter genes to successfully track gene expression in transgenic models, transfected tumor xenografts, or viral-mediated gene transfer experiments. At this time, MRS does not produce true, spatial ''pictures'', and, instead, shows spectral tracings of the various metabolites it is able to identify. It is a sensitive, quantitative, and relatively fast technique when compared to MRI (29).

One particular MRS-sensitive metabolic reaction produces ATP, a process catalyzed by creatine kinase (CK):

$$H^+ + PCr + ADP \leftrightarrow ATP + Cr \quad (1)$$

where PCr is phosphocreatine and Cr is creatine. More specifically, ^{31}P-MRS identifies amounts of phosphocreatine (PCr), ATP, ADP, and free phosphorus in the reaction. This molecule can be detected readily in the heart, muscle, and brain since they are produced in great quantities in these organs. The liver, on the other hand, has very low levels and thus can serve as a background for situations where CK is overexpressed. A transgenic mouse model that overexpresses this enzyme in the liver has shown that it can generate MRS-detectable levels of PCr (83). This technique is however invasive in nature, for a tissue window has to be created to minimize background noise from the overlying muscle and other surrounding structures rich in CK. Future developments in ^{31}P 3D spectroscopy may eventually prove helpful.

A related study employs an invertebrate analog of CK as its reporter gene. *Drosophila melanogaster* arginine kinase (AK) has been cloned and, when introduced into mammalian muscle, produces phosphoarginine (PArg) in the following reaction (84):

$$H^+ + PArg + ADP \leftrightarrow ATP + Arg \quad (2)$$

PArg provides a unique phosphorus signal that is amplified when the transgene for AK is delivered to mammalian tissues (Fig. 12). One important consideration in the use of *Drosophila* AK is it can act as an ATP buffer in mammalian tissues and, thus, the consequences of this need to be explored prior to its widespread use as an in vivo gene reporter gene.

Another MRS-friendly system is the cytosine deaminase (CD) reporter system (85). Using ^{19}F MRS, the conversion of the relatively benign 5-fluorocytosine (5-FC) to the cytotoxic agent (5-FU) driven by this enzyme can be detected. Tumor xenografts transfected to express yeast CD have been shown to produce 5-FU with MRS.

IV. STRATEGIES FOR TRACKING VEHICLES AND TRANSDUCED CELLS

The list is quite long for the array of vehicles being developed, both viral and nonviral, for the delivery of gene vectors. The details of such carriers are, of course, provided elsewhere throughout this book. There are 2 fundamentally different ways to track the biodistribution of delivered agents. One method is to track the sites of successful gene transfer/expression (various methods described in this chapter) and the other method relies on directly labeling vehicles, DNA or cells with radionuclides, fluorescent dyes, or MR-compatible contrast agents. The latter method may give better spatiotemporal information with respect to distribution of the vehicles or DNA material used for therapy, but does not give any information with regards to the success of gene transfer. The latter method has been reviewed recently and includes the direct labeling of herpes virus with ^{111}In, adenoviral knob with ^{99m}Tc, liposomes with ^{111}In, double-stranded DNA by peptide-based chelates ([^{99m}Tc]PBC), genetically modified mesothelioma cells with technitium ([^{99m}Tc]PA1-STK), myoblasts with technetium, and DNA-delivery systems with MRI-detectable, DNA-binding chelates (86–93).

For those involved in the development of novel viral or nonviral delivery vehicles, targeting and efficiency of gene transfer are primary concerns, and, thus, examples of the former method are given here. The success of exogenous gene expression is dependent on its ability to at least survive the following series of stringent events: the DNA-vehicle complex has to bind specific cell-surface receptors, undergo receptor-mediated endocytosis, survive endosomal lysis, be released from endosomal captivity, endure the cytoplasmic environment, be destined for targeted entry of the nucleus, and ultimately released from carrier molecules to facilitate gene expression (94). Whether it is pseudo-typed lentiviruses, modified PEI complexes, liposomes, etc., one major role reporter genes are expected to provide is the monitoring of localization, biodistribution, and gene transfer efficiency of these delivery vehicles in living subjects. Noninvasive localization

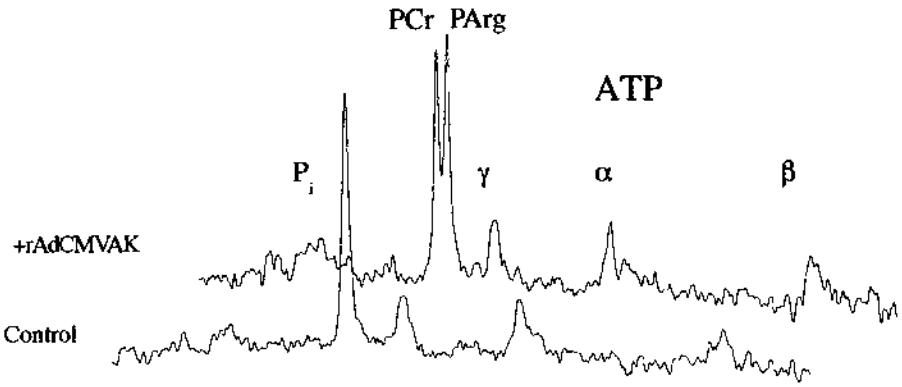

Figure 12 Magnetic Resonance Spectroscopy (MRS) of Transgene Expression ^{31}P-MRS has the ability to detect phosphorus NMR signals. A recombinant adenovirus (rAdCMVak) can be constructed to deliver arginine kinase, an enzyme unique to invertebrates, into muscle. Once introduced into mammalian muscle, the enzyme catalyzes the production of phosphoarginine (PArg), which has a unique spectral signature (both in magnitude and location on the spectrum) that can be detected by MRS. Figure represents in vivo basal ^{31}P spectra from the hind limbs of a 6-month-old mouse. ^{31}P-MRS spectra from the rAdCMVAK-injected limb (upper spectrum) reveal a ^{31}P resonance at the chemical shift for PArg that is not present in the contralateral control limb (lower spectrum). (Images reproduced with permission from Ref. 84.)

of retroviral- (95), adenoviral- (96), and herpes viral vector- (97) mediated HSV1-*tk* gene transfer has been performed. Fig. 13 is an example of replication-conditional, oncolytic herpes simplex virus-mediated gene delivery. Constitutively expressed optical and/or PET reporter genes have been helpful in studying the distribution of nonviral vehicles such as PEI polyplexes, which have been covalently modified with transferrin to facilitate targeting (Fig. 14), and cationic lipid-DNA complexes (Fig. 15).

V. MONITORING GENE THERAPY LEVELS

Precise localization and quantitative assessment of the magnitude and temporal variation of transgene expression is a necessary component of any gene therapy trial. Direct imaging with a transgene-specific imaging probe is ideal but neither feasible nor practical in most cases. To develop a specific probe for each individual transgene is not always technically possible; furthermore, it is necessarily labor and cost-intensive. Using

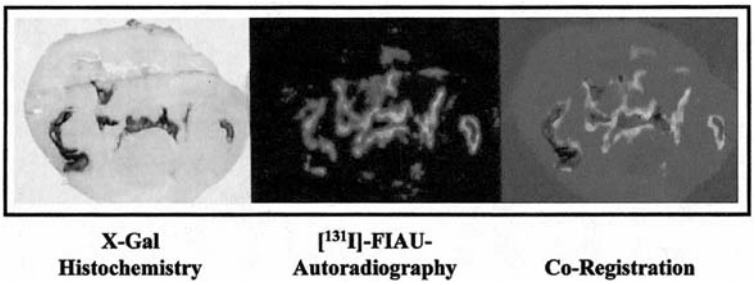

Figure 13 Tracking HSV Infection with [^{131}I]FIAU Using Autoradiography Tracking wild-type HSV-1 infection with radionuclide-based techniques can be accomplished using the virus's native thymidine kinase gene and a reporter probe such as radiolabeled [^{131}I]FIAU. To help corroborate imaging findings with histochemistry findings, a replication conditional, oncolytic recombinant HSV-1 virus vector, hrR3, containing a *lacZ* insertional mutation within the *RR* gene locus, has been prepared. Following injection of the vector into rat gliosarcoma xenografts, tumors were processed for tissue-sectioning, autoradiography, and β-galactosidase-stained histology. Image coregistration of tumor histology, HSV-1-*tk*-related radioactivity (assessed by [^{131}I]FIAU autoradiography), and *lacZ* gene expression (assessed by β-galactosidase staining) demonstrated a characteristic pattern of gene expression around the injection sites. A narrow band of *lacZ* gene expression immediately adjacent to necrotic tumor areas is observed, and this zone is surrounded by a rim of HSV-1-*tk*-related radioactivity, primarily in viable-appearing tumor tissue. PET images (not shown) of injected tumors in the intact animal have also been performed using [^{124}I]FIAU as a reporter probe; the areas of PET-labeled probe uptake correlate well with the β-galactosidase-stained photomicrographs. See color insert for color version of this figure. (Image reproduced with permission from Ref. 97.)

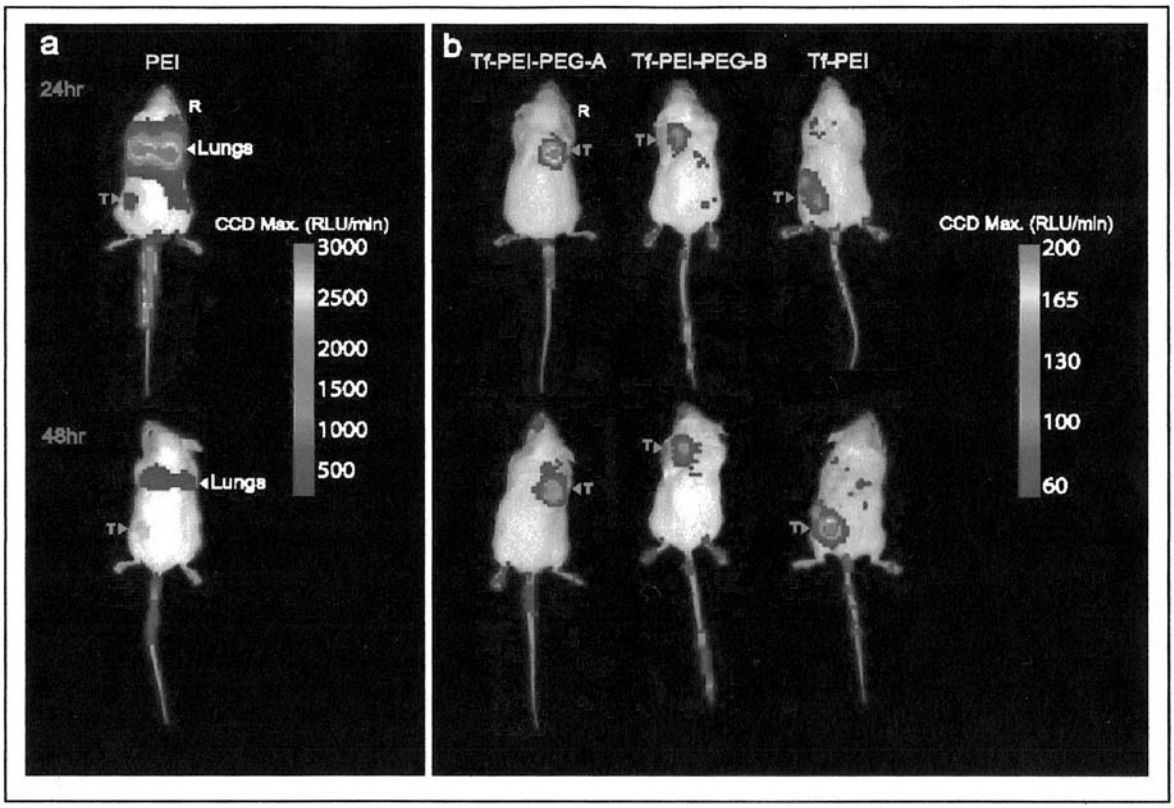

Figure 14 Tracking Transferrin Targeted Polyethylenimine (PEI)-Mediated Gene Delivery Using Optical Bioluminescence Imaging Delivery of the bioluminescence reporter gene, firefly luciferase (*Fluc*), by CMV-*Fluc* DNA/PEI polyplexes and subsequent *Fluc* expression can be imaged in living mice using a cooled CCD camera. Additionally, the biodistribution of modified PEI polycation complexes, altered with molecules such as transferrin and/or polyethylene glycol (PEG), can be studied in this manner. Transferrin targeting has been shown to improve the transfection efficiency in certain tumor cell lines, and PEG modification has been shown to improve circulation times of DNA/PEI complexes and prevent their nonspecific uptake by the reticuloendothelial system. All CCD images are of living mice carrying N2A xenograft 24 or 48 h after intravenous injection of various DNA/PEI polyplexes. Site of tumor is indicated (T). (a) PEI (positive control) treated animals show relatively high *Fluc* expression (using 1× D-Luciferin) in the lungs as compared with the tumor. The activity on the left hind limb is from the N2A cell tumor (T). Nonspecific tail activity occurs at the DNA/PEI polyplex injection site. All *Fluc* expression decreases at 48 h. (b) Tf-PEI-PEG-A-, Tf-PEI-PEG-B-, and Tf-PEI-treated mice show *Fluc* expression (using 2× D-Luciferin) in the tumor (T) and tail regions, but no detectable signal in the lungs. For each formulation, expression in the tumor varied over 24 to 48 h. All images are quantitated as indicated by the 2 scales (RLU/min). See color insert for color version of this figure. (Image reproduced with permission from Ref. 119.)

indirect imaging methods by linking a portable reporter gene to a therapeutic gene allows for more flexibility, as a variety of transgenes can be individually monitored by cloning a reporter gene into appropriate sites of the vector. Coexpression of the therapeutic gene product and reporter gene product in a coordinated and regulated manner enables a correlative and quantitative relationship between the 2 genes. Thus, levels of therapeutic gene expression can be inferred by the amount measured from reporter genes, provided that the expression of both genes remains coupled. Several such approaches, ranging from the more straightforward, like the dual vector approach, to the more sophisticated, such as the bidirectional transcriptional approach, are currently being developed and are briefly discussed below (78).

A. Covector Administration

One relatively simple method to monitor gene therapy in vivo is to coadminister 2 different vectors that are identical in every regard with the exception of the transgene they are carrying: one vector would encode the therapeutic gene, the other would encode the reporter gene, and both genes would be driven by the same promoter. This approach has been validated using the 2 PET reporter genes, HSV-sr39*tk* and D2R, each cloned

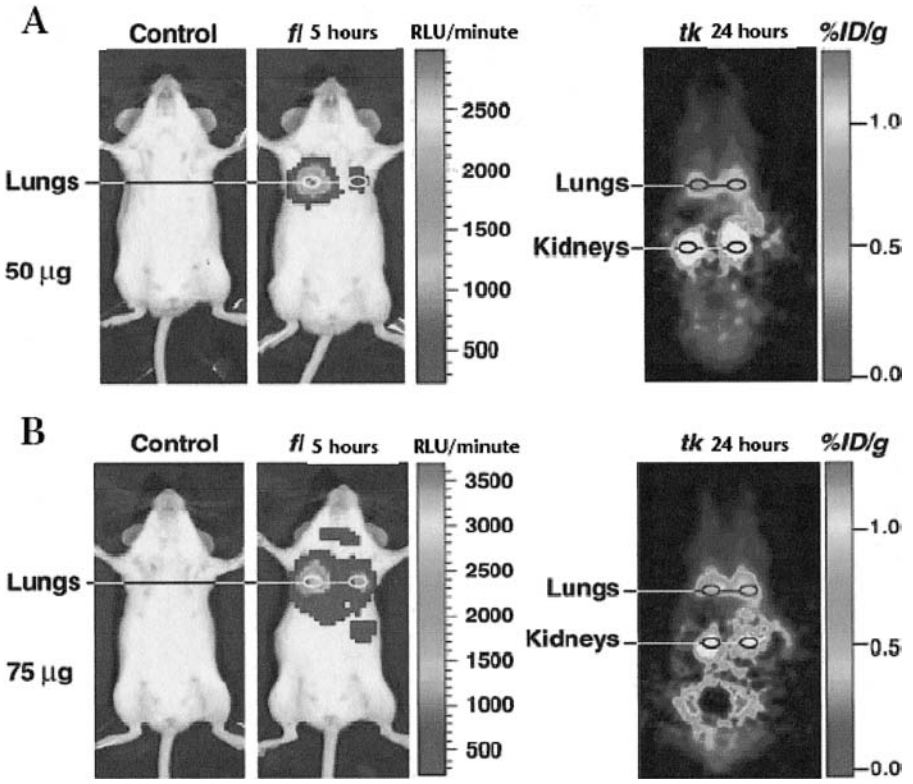

Figure 15 Tracking Cationic Lipid-Mediated Reporter Gene Delivery Using Optical (Bioluminescence) and PET Imaging Cationic lipids associate with negatively charged DNA to form complexes that bind to cell surfaces by way of electrostatic interaction, thereby allowing a nonviral means of gene transfer. Distribution of systemic administration of DNA-lipid complexes in mice is demonstrated by delivering prepared DNA-lipid complexes that carry optical and PET reporter genes. CMV-*fl* plasmid DNA (cytomegalovirus (CMV) promoter driving expression of firefly luciferase (*fl*) gene) was mixed with cationic lipid, 1,2-dioleoyl-3-trimethylammonium-propoane (DOTAP) and cholesterol, to form *fl* DNA-lipid complexes. A similar procedure was used to prepare HSV1-sr39*tk* DNA-lipid complex (*tk* DNA-lipid complex). (A, B) Figures A and B show images following administration of 50 μg and 75 μg each of *fl* and *tk* DNA-lipid complexes via tail vein injection into CD-1 mice, respectively. Bioluminescent images (left images) were obtained 5 h after injection of the vector and 5 min after intraperitoneal injection of D-Luciferin. MicroPET images (right images) were obtained 24 h after vector delivery and 1 h after [^{18}F]FHBG injection. Control mice (left) optical images were obtained prior to administration of D-Luciferin. Optical and PET images demonstrate that lungs are primary organs for transgene expression. Increased dose of DNA-lipid complex results in greater pulmonary transgene expression. Activity seen in the kidneys in the microPET images is the result of excreted, unsequestered, reporter probe, [^{18}F]FHBG. See color insert for color version of this figure. (Image reproduced with permission from Ref. 120.)

into distinct adenoviral vectors and both driven by the same CMV promoter (68). While individual cell differences in expression levels may be seen, macroscopic measurements made at the tissue culture or organ level (adnenoviral-mediated hepatic transfer) correlate quite well ($r^2 \geq 0.93$). The technique may prove useful in specific experimental situations.

B. Single Vector Approaches

The use of an internal ribosomal entry site (IRES) is a hallmark of the bicistronic approach to coupling genes (98–100). In a bicistronic expression cassette, an IRES sequence is interpositioned between the therapeutic and reporter gene, usually the first and second cistron, respectively. Both genes are under the control of the same promoter, and transcription of this construct results in a single mRNA molecule. Initiation of translation of the first cistron is by way of the usual cap-dependent manner, but translation of the second cistron is facilitated by the IRES sequence in a cap-independent mechanism, which allows binding a translation by a second ribosome. This approach has been verified in a few studies. For example, an IRES derived from an encephalomyocarditis virus has been used to construct a bicistronic vector from

which both D2R and HSV1-sr39*tk* reporter genes are coexpressed from a common CMV promoter (pCMV-D2R-IRES-HSV1-sr39*tk*) (47). The levels of D2R and HSV1-sr39tk activity demonstrate a high degree of correlation ($r^2 = 0.97$) using [^{18}F]FESP and [^{18}F]FHBG as imaging probes, respectively. Another vector that encodes Renilla luciferase (*Rluc*) in a bicistronic configuration, pCMV-*Rluc*-IRES-sr39*tk* or pCMV-sr39*tk*-IRES-*Rluc*, also shows excellent correlation (47). Similar relationships have also been seen with the use of an HSV1-*tk* gene that has been ''IRES-linked'' to the *lacZ* gene (101); imaging with iodinated FIAU (SPECT reporter probe for HSV1-*tk*) correlates well with β-galactosidase activity seen by light microscopy. These studies corroborate the use of radionuclide and optical reporter genes as a means of quantitatively determining relative levels of target gene expression.

One interesting finding in this approach, however, is that expression levels from the gene upstream to IRES sequence is consistently more robust then the levels seen from the gene downstream to the IRES (47). This may have to do with cell-specific differential translation from the IRES sequence, but, regardless, emphasizes the need for a highly sensitive reporter system, as levels of the reporter gene product will be significantly diminished compared to the upstream gene. Further understanding and exploitation of regulatory ''modules'' recently found within the IRES may help circumvent this problem in the future (102). Alternatively, 2 different genes from 2 distinct, but identical, promoters within a single vector can be expressed to avoid the attenuation problem and tissue variation issues experienced with the IRES-based approach. This is a variation of the bicistronic approach and is otherwise known as the ''dual-promoter'' approach (76). Strong correlation between 2 reporter genes, *hsstr2* and HSV1-*tk*, each driven by an independent but identical CMV promoter, has been exhibited (Fig. 10).

In some situations, gene therapists will want to externally control the levels of transgene expression and, additionally, will need to verify the extent of control with imaging techniques. One particularly novel indirect imaging method makes this entire scenario possible by the use of a single inducible bidirectional tetracycline-responsive element and 2 flanking minimal CMV promoters (Fig. 16A). The fusion transactivator protein rtetR-VP16, which is constitutively expressed and can potentially be incorporated into the same bidirectional vector, binds to TRE only in the presence of tetracycline or 1 of its analogs. By varying levels of an exogenously added inducer such as doxycycline, a gene therapist can control transcription and magnitude of expression, which can be verified by the accompanying reporter gene. Proof of principle has been shown in rat xenograft models using the reporter genes D2R and HSV1-sr39*tk* (Fig. 16B) (103).

The fusion gene/protein approach is yet another powerful means of indirect monitoring of gene therapy. Constructs in this protocol contain 2 or more genes linked together within the same reading frame so that a single protein is translated. The resultant hybrid or fusion protein will have therapeutic and reporter properties, and the expression of the fused gene can be closely monitored since the expression of the therapeutic component is stoichiometrically coupled to the reporter component of the protein. HSV1-*tk*-*gfp*, HSV1-*tk*-*Fluc*, *gfp*-*Fluc*, and HSV1-sr39*tk*-*Rluc* are a few successful examples (21,104–106). The fusion gene is engineered such that the proteins are linked via a short peptide spacer. However, while appealing in concept, it is quite challenging in practice to produce generalizable proteins, since fusion proteins are often inactive or less active than their individual counterparts; furthermore, fusion proteins also may not localize to appropriate compartments since appropriate signaling mechanisms are either masked or unavailable (78). Using this technique for every therapeutic gene developed would prove to be a daunting task, since each newly generated fusion protein has its own peculiarities. Future technological improvements in the physical linkage of 2 proteins may help minimize discrepant behavior and allow more flexibility for this technique.

VI. CLINICAL APPLICATIONS

While human gene therapy trials have been in effect for several years, the imaging of transgene expression in humans has only started. Transgenes, which can serve as both therapeutic genes and reporter genes (i.e., ''direct imaging''), will most likely have an easier transition towards clinical application when compared to indirect imaging methods. More specifically, the kinetics, biodistribution, stability, dosimetry (needed for radionuclide approaches), and safety of the transgene and reporter probe have to be determined in direct-imaging reporter protocols. In comparison, an additional verification and characterization of the reporter gene product has to be performed in indirect imaging systems.

Utilizing a direct-imaging method, such is the case with the HSV1-*tk* suicide/reporter gene and one of its reporter probes, [^{18}F]FHBG, where the requisite pharmacokinetics, biosafety, and other related testing have been established in human volunteers (55). The study indicated that while [^{18}F]FHBG demonstrates acceptable characteristics and should prove to be an acceptable probe for HSV1-*tk* imaging, [^{18}F]FHBG does not readily cross the BBB. Methods used to disrupt the BBB during [^{18}F]FHBG administration may aid in this regard if needed. Furthermore, imaging HSV1-*tk* reporter gene expression near the gall bladder, kidneys, and bladder with [^{18}F]FHBG may prove to be difficult since nonsequestered [^{18}F]FHBG passes through these organs, contributing to the background signal seen in these structures. Clinical trials with [^{18}F]FHBG in patients undergoing HSV1-*tk* gene therapy have recently begun.

In another study, which also employs a suicide/reporter gene system, a small group of patients suffering from recurrent glioblastomas have been treated with suicide gene therapy (HSV1-*tk*) (Fig. 17) (107). The construct has been delivered with a cationic liposomal vector and among the 5 patients treated with this therapy, 1 shows accumulation of the reporter probe [^{124}I]FIAU in a portion of the tumor. Following a 2-week course of ganciclovir, FDG PET, and methionine (MET), PET scans at the end of treatment indicate that necro-

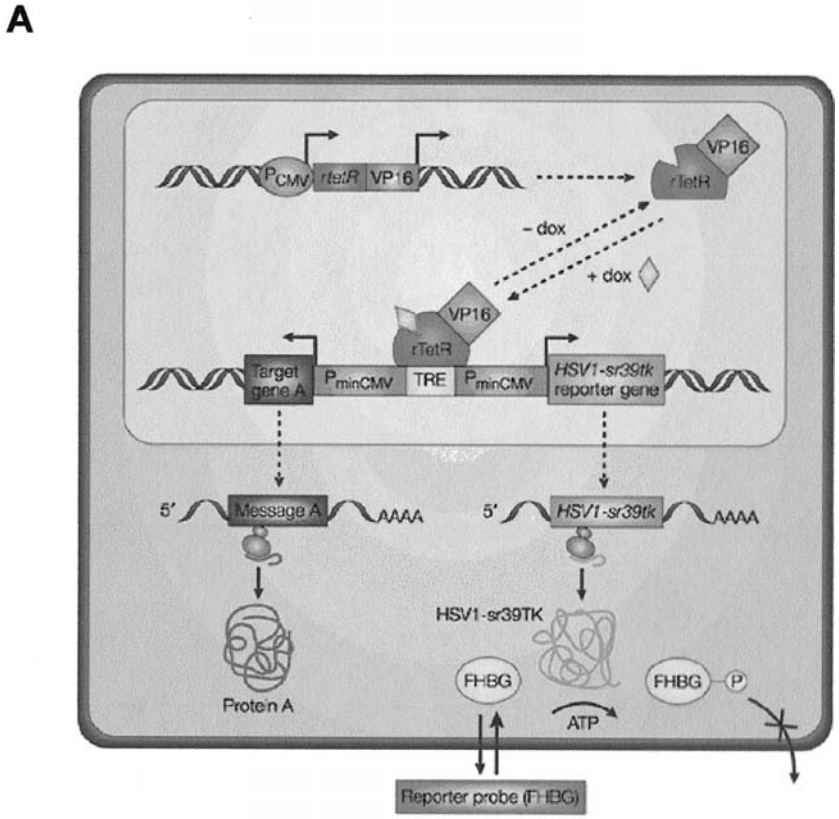

Figure 16 Indirect PET Imaging Using a Bidirectional Transcriptional Approach (A) Target (therapeutic) gene expression can be measured indirectly by imaging reporter-gene expression if expression of the 2 genes is 'linked.' Both genes can be simultaneously expressed from 2 minimal cytomegalovirus (CMV) promoters that are regulated by a single bi directional tetracycline-responsive element (TRE). The rTetR–VP16 fusion protein is produced constitutively from a CMV promoter. When the rTetR–VP16 fusion protein binds to doxycycline, this complex binds to the TRE regulatory sequence and substantially enhances expression from the 2 minimal CMV promoters. The target gene *A* in 1 coding region and a reporter gene (for example, a reporter kinase such as HSV1-sr39*tk*) in the alternative coding region are transcribed simultaneously into 2 mRNA molecules. Translation of the 2 mRNA molecules yields two distinct proteins in amounts that are directly correlated with each other. (B, see pg. 474) Quantitative imaging of the location(s) and magnitude of PET reporter-gene expression by trapping of a PET tracer inside the cell (for example, by phosphorylation of [^{18}F]FHBG by the HSV1-sr39TK reporter protein) provides an indirect measure of target-gene expression. Sequential microPET (positron-emission tomography) imaging studies of a nude mouse carrying 4 tumors. Four tumor cell lines—2 positive controls (constitutive reporter-gene expression), 1 negative control, and 1 inducible line (reporter-gene expression induced by doxycycline)—were injected subcutaneously into 4 separate sites in a single mouse. When tumors reached a size of at least 5 mm, the mouse was imaged with 9-(4-[^{18}F] fluoro-3-hydroxymethylbutyl)guanine ([^{18}F]FHBG). Doxycycline was then added to the water supply for 7 days. The mouse was then scanned again with [^{18}F]FHBG. Doxycycline was removed from the water supply for the next 7 days, and the mouse was again scanned with [^{18}F]FHBG. The locations of the 4 tumors and the mouse outline are shown by the dotted regions of interest. All images are 1–2 mm coronal sections through the 4 tumors. The % ID/g (% injected dose per gram tissue) scale for [^{18}F]FHBG is shown on the right. The negative control tumors show no gene expression, and the positive control tumors show increased expression over the time course. The tumor on the top right, with inducible gene expression, initially does not accumulate [^{18}F]FHBG, then at 7 days after addition of doxycycline, induction of reporter-gene expression traps [^{18}F]FHBG. Seven days after withdrawal of doxycycline, there is decreased induction and minimal trapping of [^{18}F]FHBG. The [^{18}F]FHBG image signal correlates well with target-gene expression (not shown). See color insert for color version of this figure. (Images reproduced with permission from Ref. 23.)

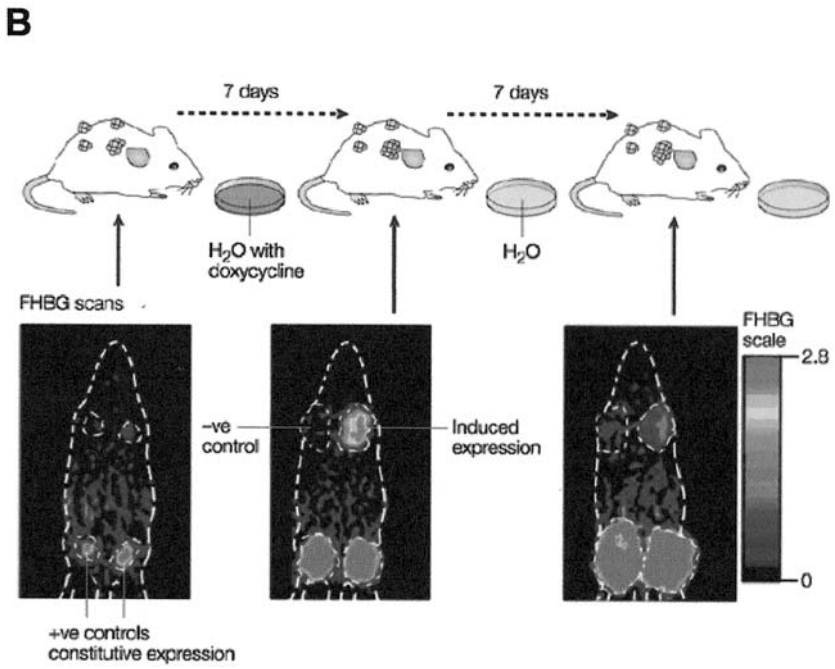

Figure 16 Continued.

sis occurs in the region where [^{124}I]FIAU uptake was previously shown, suggesting successful suicide gene transfer to a portion of the tumor. Those patients who could not accumulate detectable levels of [^{124}I]FIAU in their infused tumors were found to have a low mitotic index in their tumors.

With this small yet extremely exciting study, we are able gain a significant amount of knowledge about human gene therapy using in vivo imaging methods. For example, the investigators were able to identify and localize successful gene transfer in 1 patient 68 h after tumor infusion. Also, in the patient where [^{124}I]FIAU uptake was seen, only a fraction of the tumor was sequestering [^{124}I]FIAU. This suggests that the rest of the tumor was either not transduced or that the transduction was too weak to detect. Additional follow-up scans of the patient would help to determine the outcome of the heterogeneously treated tumor as well as the importance of reporter-probe uptake. In the patients that did not demonstrate [^{124}I]FIAU uptake, the failure to see [^{124}I]FIAU reporter-probe accumulation may be due to sheer lack of transducible cells or failure of gene transfer. Future studies of these and other issues are certain to be extremely helpful in understanding the effectiveness of gene therapy.

VII. DECISION ALGORITHM FOR REPORTER GENE TECHNOLOGY

The road to clinical gene therapy trials is an arduous one but can be helped with the variety of reporter-gene technologies described in this chapter. As with any drug testing, there is a prerequisite period of preclinical testing, much of which takes place in animal models. As expected, there will be a strong inclination towards optical and radionuclide approaches, since these approaches are the most accessible and more rigorously tested among the imaging options. Furthermore, it is likely that a single investigator or a single group of investigators working on a specific gene therapy will most likely have to utilize a combination of modalities (e.g., optical *and* radionuclide-based technologies) to efficiently test gene-therapy vectors and to facilitate their use in higher organisms. As testing progresses in higher species, efforts need to be made toward the radionuclide approaches, and substantial investment of time and money may be needed to develop PET-reporter probes. Eventually, human trials will employ PET, Gamma/SPECT, and, in the future, MR-based methods. The decision and timing to use certain reporter systems over others is a relatively complex one, and we will attempt to simplify the decision-making process by providing the set of guidelines we use to make such decisions.

The first decision depends upon whether an investigator is attempting to study the biodistribution/pharmacokinetics of a gene-delivery vehicle or whether one is interested in monitoring gene expression. From an earlier discussion (see Section IV), the distribution of a vehicle can be directly imaged by directly labeling the vehicle with radioisotope, fluorescent, or MR-compatible markers. Distribution of injected vehicle can also be inferred from a reporter gene coupled to a constitu-

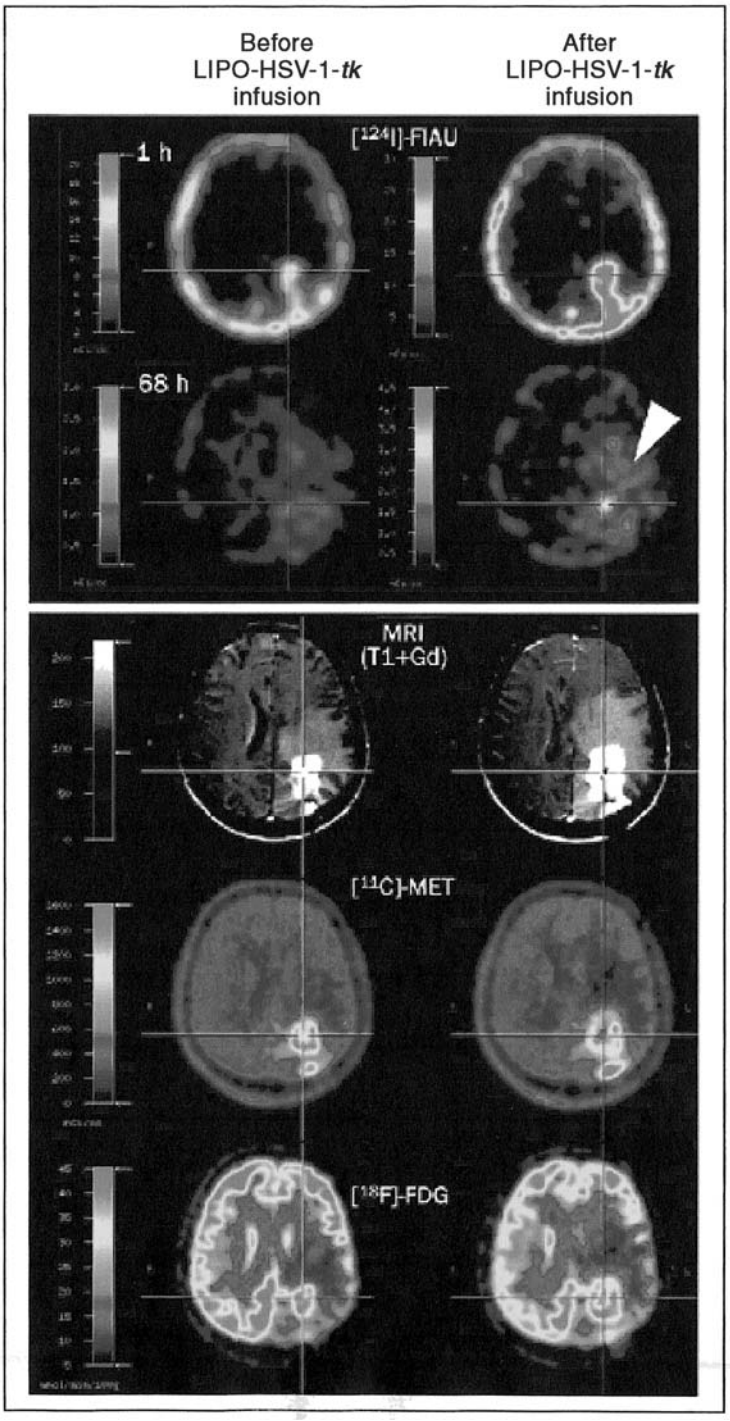

Figure 17 Human Brain Tumor HSV1-tk Suicide Gene Therapy Using Direct PET Imaging PET brain imaging of HSV1-*tk* suicide gene therapy in a patient with [^{124}I]FIAU as a reporter probe. A single, representative transverse image of a brain from a patient suffering from recurrent glioblastoma pre- and post-gene therapy treatment. Coregistration of [^{124}I]FIAU-PET, MRI, [^{11}C]methionine (MET)-PET, and FDG-PET before and after intratumorally infused liposome-gene complex containing HSV1-*tk* (LIPO-HSV-1-*tk*). The white arrow marks the region within the tumor where specific [^{124}I]FIAU retention was imaged after LIPO-HSV-1-*tk* transduction (2nd row, 2nd column). The cross hairs in the right column indicate signs of necrosis after ganciclovir treatment (5 mg/kg twice a day over 14 days) that was started 4 days after vector application. The area of necrosis in the tumor as depicted by reduced methionine ([^{11}C-MET]) uptake and decreased glucose [FDG] metabolism. See color insert for color version of this figure. (Images reproduced with permission from Ref. 107.)

tive promoter. However, this method is less sensitive since the rate of gene transfer is always less than 100%, and often significantly lower.

For investigators interested in monitoring transgene expression, a search for reporter probe(s) that may already exist for the transgene is mandatory. For example, for those who use the suicide gene, HSV1-*tk*, a number of reporter probes already exist, and direct-imaging protocols can be performed using radionuclide techniques. In the example of HSV1-tk, the decision to use [^{124}I]FIAU over other probes such as [^{18}F]FHBG is based on preliminary favorable evidence towards [^{124}I]FIAU described earlier. However, the selection of the appropriate reporter probe is not trivial, and, as further tests are being performed, it is likely that the other reporter probes may prove to be just as or more efficient than FIAU, depending on specific cell-type, local pharmacokinetics, mode of delivery of the transgene (viral vs. nonviral), etc. As we learn more about each reporter system, selection of specific transgene-reporter probe combinations will be disease-specific; it is likely that each reporter combination will have certain strengths and weaknesses depending on the disease model and target tissues. The investigator(s) will have to adjust accordingly. Future studies are bound to address these and other related issues.

If no reporter probe for the transgene exists, then indirect-imaging methods are needed. This requires the coupling of the therapeutic gene(s) with an optical, radionuclide-based or MR-based, reporter gene(s). As described earlier, a number of indirect methods are available, including the dual promoter approach, bidirectional approach, bicistronic approach, etc. The selection of 1 of these promoter configurations will largely depend upon the nature of the promoter of the therapeutic gene. If a robust, constitutive promoter drives the therapeutic gene, then the dual promoter, the bicistronic (IRES-mediated), or bidirectional approach may suffice. However, if a weak or tissue-specific promoter drives the therapeutic gene, then amplification strategies for either the reporter gene (and possibly the therapeutic gene) will need to be employed. Amplification strategies for tissue-specific promoters using the VP16 transactivating domain fused to the yeast GAL4 DNA-binding domain has been verified for use with reporter genes (108).

A variety of other factors also dictate the selection of reporter gene systems. For example, if a research group is intent on bringing a specific gene therapy to human application, it behooves them to use multimodality reporter-gene systems, i.e., use fused reporter genes such as HSV1-*tk*-*Fluc* (keeping in mind, HSV1-*tk* is *not* being used as a therapeutic gene in this case when the reporter probe is administered in nonpharmacological amounts). By coupling a therapeutic gene to a fused reporter gene, it will be possible to move quickly between the preclinical verification phase (optical imaging of Firefly luciferase) to the clinical phase (radionuclide-based imaging of HSV1-*tk*). Alternatively, if a research group strictly deals with small animals, then the use of optical reporter genes only may suffice. Along this line of reasoning, the use of large animals precludes the use of optical methods, and as a result, radionuclide-based or MR-based technologies become more important.

Imaging disease processes in the central nervous system (CNS) also limits the selection of reporter genes. Some of the radionuclide-based technologies, such as HSV1-*tk* and its mutants, are limited since their reporter probes do not readily cross the blood-brain barrier (BBB). On the other hand, [^{18}F]FESP, the reporter probe for D2R, easily crosses the blood-brain barrier and can be used to a certain extent in the CNS. Use of the D2R-[^{18}F]FESP system in the CNS is limited in the striatum where endogenous dopamine receptors are present. Optical methods, both fluorescence and bioluminescence, can be utilized if specific spatial localization is not needed. The substrates of the bioluminescence methods, D-Luciferin and coelenterazine, easily cross the BBB, which facilitates the use of these reporter genes in the CNS.

By the same token, imaging in the lung, connective tissue, or cortical bone may limit the use of superparamagnetic-labeled agents in MR imaging since it will be difficult to differentiate between the signal from the contrast agent and the signal of these anatomical structures, since they are identical in certain MR sequences.

Other important factors in the selection of reporter genes include the need for good spatial resolution (preference given to radionuclide and MRI-based imaging), repetitive imaging (preference given to bioluminescence and most radionuclide approaches), and image quantitation (preference given to radionuclide, optical, and MRS-based methods). Cost, institutional infrastructure, requirement for support personnel, and the physical space required can also be significant factors that favor the optical and gamma camera methods and less so towards the PET and MR-based methods. Thus, a large number of factors have to be considered prior to the selection and implementation of reporter genes in living subjects. With careful planning, an optimal imaging strategy can be followed and be enormously helpful in the study of gene therapy and disease entities.

VIII. SUMMARY AND FUTURE DIRECTIONS

A wide array of tools is becoming available for the evaluation of gene therapy in living subjects. These tools will be extremely helpful towards the advancement of human gene therapy by providing a means of continuous monitoring of location(s), magnitude, and temporal variation of gene delivery and expression. Viral and nonviral transfer of genetic material is bound to improve, and these developments will be supported by direct and indirect reporter-gene technologies. The ability to monitor gene expression through a variety of bicistronic vectors and to control gene expression, either through inducible promoters and/or through tissue-specific amplification techniques, is likely to play an increasing role in gene therapy as we begin to move away from constitutive expression in some cases.

In the next decade, PET/CT and SPECT/CT will probably be the major workhorses for human gene therapy trials. PET-

based technologies particularly have an advantage given their greater sensitivity as well as the ability to use biological molecules that nearly mimic its the parent molecule after being radiolabeled. Future technical improvements are certain to aid optical and MR-based protocols for human application. Continued alliances between gene therapists, molecular biologists, engineers, chemists, physicists, and pharmacologists will help build the next generation of molecular imaging technologies to expand its capabilities in gene therapy.

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24

Gene Transfer into Hematopoietic Stem Cells

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The goal of this chapter is to describe the properties of hematopoietic stem cells (HSC) and the introduction of novel genetic material into HSC. The purification, phenotype, and analysis of mouse and human HSC will be reviewed as will animal models that demonstrate the potential and the risks of gene therapy. Finally, some of the problems confronting gene therapy for diseases of the hematopoietic system will be discussed. The work described in this chapter comes from many different fields of study to which many authors and laboratories have contributed. The references cited should be considered to be representative, not complete.

I. PROPERTIES OF HEMATOPOIETIC STEM CELLS

The hematopoietic stem cell (HSC) is the ultimate progenitor of all of the cells found in the peripheral blood. In mammalian systems, small numbers of HSC have been shown to be capable of extensive proliferation, generating millions of mature blood cells in regulated numbers each day. HSCs are multipotent and differentiate into cells of the erythroid (red cell), megakaryocytic (platelets), myeloid (granulocytes and monocytes), and lymphoid (B- and T-cell) lineages (Fig. 1). HSC can self-renew without differentiating, generating pluripotent progeny, which themselves can proliferate and differentiate into mature blood cells, [for reviews see (1–4)]. The ability of HSCs to self-renew allows the transplantation of a small number of HSCs to reconstitute the entire hematopoietic system of patients whose bone marrow had been ablated by chemotherapy or radiation (5–7).

The hematopoietic stem cell is especially attractive as a target for gene therapy of hematopoietic diseases (8–10). In theory, a small number of HSCs could be exposed to gene transfer vectors ex vivo and returned to a myeloablated recipient. Repopulation of the recipient with gene corrected HSC would ensure a lifetime supply of modified peripheral blood cells of all lineages. If the transferred gene (transgene) were expressed at the appropriate levels in mature hematopoietic cells carrying the transgene, gene therapy could be the treatment of choice for many inherited and acquired hematopoietic diseases (8–10). In addition, recent evidence has suggested that the most primitive mouse bone marrow cells can give rise to cells of the vascular endothelium, and perhaps to many solid tissues as well (11–14). Thus the therapeutic potential of HSC may extend beyond the treatment of hematopoietic diseases. At this point, the experiments suggesting that primitive hematopoietic cells can directly transdifferentiate into functional cells other than the vasculature should be considered exciting but preliminary, as alternate explanations are possible (15).

As with any novel therapy, a careful examination of possible negative outcomes should also be considered. Many hematopoietic malignancies arise as a result of mutations in primitive hematopoietic cells with great proliferative potential. In fact, many of the gene transfer vectors used to deliver new genetic material to HSC are derived from viruses first identified as pathological agents (16,17). The many modifications made to these viruses to negate possible pathological consequences is the subject of other chapters, but it should be remembered that even the most highly engineered vector still contains viral elements that can cause disease.

II. MOUSE HEMATOPOIETIC STEM CELLS

A. Transplantation Assays

The definitive assay for mouse hematopoietic stem cells is the repopulation of stem cell deficient mice with transplanted hematopoietic cells (1,3,4). Repopulation by donor cells, as opposed to repopulation by residual host cells, is demonstrated

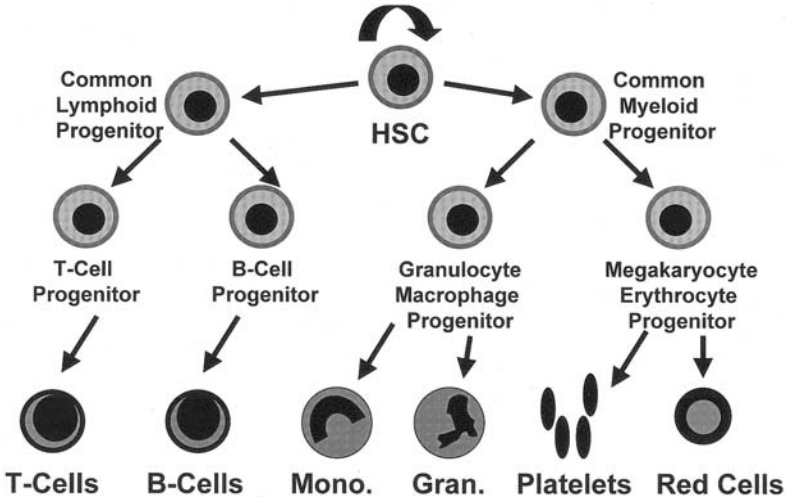

Figure 1 A model of hematopoiesis. Hematopoietic stem cells (HSC) can either self-renew or give rise to progenitor cells committed to either myeloid (CMP) or lymphoid (CLP) differentiation. As differentiation progresses, hematopoietic cells proliferate (not shown) and become progressively restricted to specific lineages of cells.

by analysis of genetic markers that differ between the donor and recipient mouse. The genetic markers used include chromosomal translocations (18), isozyme polymorphisms (19,20), Y-chromosome-specific DNA sequences (21), cell surface markers (22), and combinations of these. Multilineage repopulation of lethally irradiated hosts is demonstrated by the detection of the host genetic marker in cells of the different hematopoietic lineages (3,4).

The repopulation of recipient mice with limiting numbers of bone marrow cells has shown that HSC are a rare population of cells. For example, 1 study transplanted bone marrow cells from female mice heterozygous for the X-linked isozyme marker Phosphoglycerate Kinase (Pgk) into stem-cell-deficient W/W^v mice (19,23). Due to random inactivation of 1 X chromosome, individual HSC from heterozygous Pgk-a/b mice express either Pgk-a or Pgk-b. The peripheral blood cells of mice repopulated with large numbers of bone marrow cells contained an equal amount of Pgk-a and Pgk-b. In animals repopulated with successively fewer HSC, the relative contribution of individual HSC becomes greater. As predicted, the peripheral blood cells of individual mice repopulated with limiting numbers of bone marrow cells contained high levels of either Pgk-a or Pgk-b. This work and related studies demonstrated that a single HSC could repopulate a mouse, but that repopulation required an average of 1×10^5 bone marrow cells (19,20,24). By contrast, limiting dilution assays have shown that the concentration of the repopulating stem cell in the bone marrow is more than 10-fold less than the concentration of the Colony Forming Unit-Spleen (CFU-S) (25), a multipotent cell that forms a mixed colony in the spleen of irradiated mice. The concentration of repopulating stem cells is also 10-fold less than the cell that is required for the 30-day survival of irradiated mice (3).

Competitive repopulation assays, in which mixtures of genetically distinguishable hematopoietic cells injected into stem-cell-deficient mice allow the relative ability of each population of cells to repopulate recipient animals can be quantified (26,27). These assays are a powerful tool for identifying, characterizing, and quantifying HSC.

B. Phenotype of Mouse Hematopoietic Stem Cells

The advent of Fluorescence Activated Cell Sorting (FACS) and the development of monoclonal antibodies that recognize specific markers expressed on the surface of hematopoioetic cells have made it possible to separate the rare HSC from the large number of more mature hematopoietic cells. Spangrude et al. (22) demonstrated that mouse HSC do not express antigens present on surface of mature cells of the different hematopoietic lineages. Lineage marker negative (Lin $-$) cells represent less than 10% of bone marrow cells. Further enrichment of HSC was achieved by selecting Lin $-$ cells expressing low levels of Thy-1.1 and the Sca-1 (Stem Cell Antigen) marker. Lin $-$ Thy-1.1^{lo} Sca-1 $+$ cells comprise less than 1% of Lin $-$ cells and are highly enriched for HSC and other primitive progenitor cells, including CFU-S and radioprotective cells (22).

The Sca-1 marker is expressed in about 50% of all inbred mouse strains, and the Thy-1.1 allele is present in only a few inbred strains (28). The Sca-1 and Thy-1.1 markers are found together in only 1 strain of mice (29). Several groups have shown that c-kit, the receptor for the hematopoietic growth factor SCF (Stem Cell Factor), can be used to discriminate between HSC and more mature hematopoietic cells (30–34). In most strains of mice, Lin $-$ cells expressing high levels of

c-kit, (c-kit^{HI}) are highly enriched for HSC (30). In strains expressing Sca-1, the Lin− c-kit^{HI} cells are also Sca-1 positive (33). HSC can also be distinguished from primitive progenitors that give rise to myeloid or lymphoid cells, the common myeloid progenitor (CMP) (35) and common lymphoid progenitor (CLP) (36) respectively. CMP are Lin− and c-kit+, but do not express Sca-1 or the Interleukin 7 receptor (IL-7R−). CLP are Lin−, c-kit+ and IL-7R+. The most accurate and widely accepted phenotype for mouse HSC is Lin−, IL-7R−, c-kit+, Sca-1+ (Fig. 1). An average of 30–50 cells enriched using these markers will repopulate 100% of recipient mice (3,4).

The fluorescent dyes Rhodamine 123 and Hoechst 33342 are pumped out of cells by ABC transporter proteins, primarily ABCG2, which is expressed at high levels in HSC (37–40). Many groups have used the Rhodamine 123 dye in combination with other markers to identify primitive hematopoietic cells, and Lin− cells that retain low levels of Hoechst 33342 (Ho^{LO}) and Rho 123 (Rho^{LO}) are enriched for HSC (41–43). A novel method to enrich HSC analyzes the blue and green fluorescence of bone marrow cells stained with Hoechst 33342 only. A rare ''side population'' of cells with low levels of blue and green fluorescence has been shown to be highly enriched for HSC (44). Side population cells express c-kit and Sca-1, and are Lin− (44). Finally, using a fluorescent substrate for aldehyde dehydrogenase (ALD), Jones et al. demonstrated that a population of small, Lin−, hematopoietic cells that express high levels of ALD were highly enriched for HSC (45). Injection of an average of 10 small Lin− ALD positive cells led to the repopulation of 100% of recipient mice.

C. Sources of Mouse Hematopoietic Stem Cells

During mouse development, hematopoiesis begins in the yolk sac at day 8.5 of the 21-day gestation period (23,46,47). Yolk sac hematopoiesis generates only nucleated erythrocytes containing embryonic hemoglobin. Yolk sac hematopoietic cells are capable of repopulating chemically ablated newborn mice (48,49), but do not repopulate adult animals (50). The first HSC capable of repopulating lethally irradiated adult mice are found in the aorta-gonad-mesonephros (AGM) region of mouse embryos at day 11.5 of gestation (50). AGM region HSC are negative for lineage specific markers and express c-kit (50). From day 12.5 to day 17.5 of gestation, the fetal liver becomes the site of hematopoiesis, producing mature erythroid, myeloid, and lymphoid cells. Fetal liver HSC are lineage marker negative, express c-kit, and the marker AA4.1 (51). After day 17.5, the fetal spleen becomes the primary site of hematopoiesis (23), and by the time of birth, the bone marrow has become the primary site of hematopoiesis. In adult mice, HSC are found in the bone marrow, spleen, and peripheral blood (23,52–54). Competitive repopulation assays have been used to estimate that approximately 80% of the HSC are found in the bone marrow, 19% of the HSC reside in the spleen, and less than 0.5% of HSC are found in the peripheral blood (53,54).

The relative and absolute number of HSC in the peripheral blood of mice can be manipulated by treatment of animals with either hematopoietic growth factors or anti tumor agents such as cyclophosphamide (CP). The redistribution of HSC and progenitor cells into the peripheral blood is termed mobilization (55). For example, in mice treated with Granulocyte-Colony Stimulating Factor (G-CSF) for 7 days, approximately 10% of the HSC are found in the bone marrow, 88% of the HSC reside in the spleen, and 2% of HSC are found in the peripheral blood (53,56,57). Mice treated with FLT3 ligand (FL) and G-CSF for up to 10 days showed greater than 200-fold increases in the repopulating ability of the peripheral blood compared to normal mice (58–60). Eight days after treatment with CP, the relative number of HSC in the peripheral blood is increased nearly 30-fold to a level similar to the HSC content of untreated bone marrow (61). Combinations of cytokines have even more pronounced effects on the level of peripheral blood stem cells, particularly in splenectomized mice. Treatment of splenectomized mice with G-CSF and SCF for 5 days causes a 3-fold increase in the total number of HSC, with 81% of the repopulating ability in the peripheral blood and 19% in the bone marrow (56,57). Similar results have been described with numerous other cytokines and cytotoxic drugs (58–61). An unexpected finding in mice treated with G-CSF and SCF was a greater than 10-fold increase in the repopulating ability of the bone marrow 14 days after cytokine treatment was discontinued (62).

III. HUMAN HEMATOPOIETIC STEM CELLS

A. Clinical Transplantation Models

The ability of human HSC to repopulate all hematopoietic lineages has been demonstrated by the success of bone marrow transplantation for the treatment of hematological diseases. Since the initial descriptions of successful bone marrow transplants (6,7), there have been many improvements in the way the recipient's bone marrow is ablated and the management of histocompatibility differences between the donor and the recipient. These developments have made bone marrow transplantation a common treatment for many inherited and acquired hematological diseases (63). Similar to studies in mice, the properties of human HSC can be analyzed in selected patients transplanted with genetically distinguishable donor hematopoietic cells (64). Through the use of isozyme polymorphisms, DNA polymorphisms, and sex chromosome differences, it has been shown that the donor bone marrow can repopulate the lymphoid, myeloid, and erythroid lineages of human recipients. In a classic example, analysis of transplant recipients infused with bone marrow from female donors heterozygous for the X-linked isozymes glucose-6-phosphate dehydrogenase (G-6-PD), phosphopglycerate kinase (PGK), or hypoxanthine phosphoribosyltransferase (HGPRT) has shown that human hematopoiesis can be reconstituted from a limited number of HSC (64).

Several sources of human HSC have been used successfully for transplantation. While bone marrow and mobilized

peripheral blood are the most common sources of HSC, fetal liver and cord blood HSC are also used [for reviews see (65–67)]. Human HSC are mobilized into the peripheral blood by treating the donor with cytokines such as G-CSF alone or in combination with other growth factors. Apheresis of the donor after 5–7 days of treatment gives a very high yield of cells, which generally exceeds the average number of stem and progenitor cells that can be harvested from bone marrow (67). Cord blood collected after delivery has also been shown to be a rich source of transplantable HSC. Recent work has shown that the HSC content of approximately 100 mL of cord blood is sufficient to repopulate 80-kg recipients (66).

B. In Vitro Assays for Human HSC

The development of in vitro assays for the most primitive human hematopoietic cells has greatly facilitated the study of human hematopoiesis. The long-term bone marrow culture (LTBMC) (68,69) and the ''extended'' LTBMC (70) are initiated by growing an adherent layer of stromal cells consisting of fibroblasts, endothelial cells, and macrophages. The stromal layers are then seeded with bone marrow cells to start the bone marrow culture. At biweekly intervals, a portion of the culture medium is replaced, and the nonadherent cells in the aspirated medium can be analyzed for myeloid progenitor colony formation. Human long-term bone marrow cultures initiated by single cells generate myeloid-colony-forming cells for periods of 40–60 days (71). After several weeks of culture, the standard LTBMC medium can be replaced with medium that supports the growth of lymphoid progenitor colonies. Following the medium change, the same culture will begin to produce lymphoid progenitor cells (72). In the extended LTBM, the remaining hematopoietic cells are reseeded onto a fresh layer of stromal cells where hematopoiesis continues for another 50–60 days. The continuous generation of hematopoietic progenitor cells over a long period of time from the extended LTBMC suggests strongly that these in vitro assays are good surrogates for the transplant experiments used to study mouse HSC (70,71).

Clonal hematopoiesis in vitro has been demonstrated using long-term culture initiating cell (LTCIC) assay (69). In this assay, hematopoietic cells are enriched for HSC, and single cells are cultured on preexisting stromal layers and analyzed for proliferation and colony formation for 40–60 days. In mouse models, the behavior of purified murine HSC has been compared in both in vivo transplant models and in LTCIC. The number of LTCICs and the number of repopulating HSC were directly proportional (73). These studies suggest that human LTCIC assays are also recognizing the most primitive hematopoietic cells. In the extended LTCIC assay, hematopoietic cells are replated onto a ''fresh'' layer of stromal cells, which are cultured for an additional 40–60 days. The cells that have the capacity to generate colony-forming cells in the extended LTCIC assay have the most primitive phenotype, and the rate at which LTCICs are marked by transduction with retrovirus vectors closely resembles the rate at which human HSC are marked with retrovirus vectors (70).

C. Immune Deficient Animal Models

Attempts to develop an in vivo transplantation assay for human hematopoietic stem cells have focused on immune-deficient sheep [reviewed in (74,75)] or mice [reviewed in (76,77)] as recipients for human hematopoietic cells. During development, the sheep hematopoietic system undergoes a rapid expansion between days 50 and 60 of gestation, while the sheep immune system does not become functional until days 67 to 77 of gestation. The window between days 55 and 63 of gestation provides an opportunity to introduce human hematopoietic cells into fetal sheep. The transplantation of human cells during the ''expansion'' period facilitates engraftment, and the presence of human cells in the fetal sheep before the immune system becomes active induces tolerance to human antigens [Fig. 2; (74,75)].

In a large series of sheep generated over the last 10 years, approximately 70% of the animals transplanted with human fetal liver cells had human hematopoietic cells in their peripheral blood and bone marrow. The human cells accounted for approximately 5% of the total number of peripheral blood and bone marrow cells, and all lineages were represented. Human cells were identified at all time points for periods of up to 4 years (78–82). To evaluate HSC self-renewal, bone marrow cells from primary animals are transplanted into preimmune fetuses. In approximately 1/3 of the recipients, human cells were detected demonstrating self-renewal of the original engrafted cells (82,83).

Human bone marrow or cord blood HSC can also engraft into fetal sheep. Approximately 50% of recipient sheep transplanted with human marrow or cord blood cells show long-term persistence of human cells. The levels of human cells in sheep transplanted with either bone marrow or cord blood HSC were as good (bone marrow) or better (cord blood) than that seen in the recipients of human fetal liver cells (84–86). Approximately 80% of these recipients showed signs of Graft vs. Host Disease (GVHD), as might be expected from transplants including mature human T-lymphocytes (84–86). The transplantation of T-cell-depleted bone marrow or cord blood prevented GVHD, but was associated with lower levels of engraftment and a lower percentage of animals engrafted (87). These observations are nearly identical to what are observed in human bone marrow transplant recipients and validate the fetal sheep model as an assay for the most primitive human hematopoietic cells.

The low percentage of human cells in the chimeric sheep can be increased by the injection of recombinant human cytokines. Injection of human Interleukin-3 (IL-3) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), or injection of Stem Cell Factor (SCF) increased the number of human cells in the blood or marrow, 5- and 2-fold respectively (82,83).

Although the fetal sheep model satisfies all of the criteria for a xenograft model for human hematopoiesis, the sheep model is not practical for most research laboratories. As a result, many groups have sought to develop small animal models for human hematopoiesis that combine the advantages

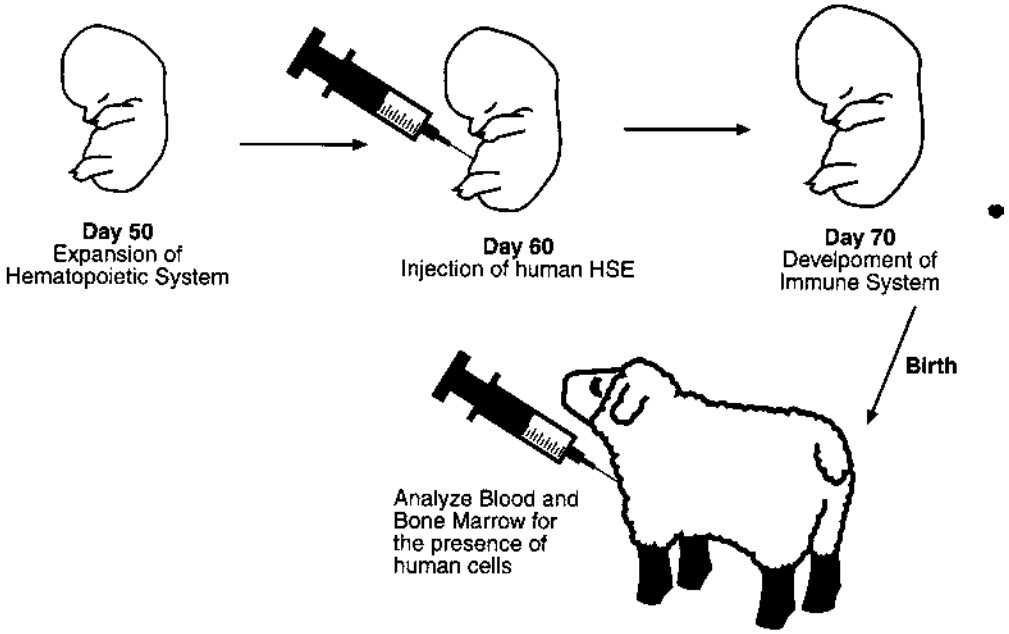

Figure 2 The fetal sheep model for the engraftment of human hematopoietic stem cells. The hematopoietic system of the developing sheep begins a rapid expansion around day 50 of gestation, but the immune system does not begin to develop until around day 70 of gestation. Injection of human hematopoietic cells around day 60 of gestation leads to engraftment and expansion of human cells that can be recovered from newborn and older sheep.

demonstrated in the fetal sheep model with the cost effectiveness of a small animal model. These efforts have focused on either beige, nude, xid (BNX) mice or mice homozygous for the severe combined immunodeficiency (scid) mutation (reviewed in (88–90).

BNX mice are homozygous for 3 mutations causing immune deficiency in the mouse. The combination of the natural killer cell deficiency caused by the *beige* mutation, the lack of a thymus caused by the *nude* mutation, and the loss of some B-cell functions caused by the *xid* mutation renders BNX mice almost completely immune-deficient. In the original report, human bone marrow cells were injected into sublethally irradiated BNX mice (91). The recipient mice contained low levels of human cells in the bone marrow and spleen (<1%), which could be detected by the presence of human repetitive DNA in these organs. In addition, human colony-forming cells from the same tissues could be identified by their selective growth in medium supplemented with human IL-3 and GM-CSF. Since hIL-3 and hGM-CSF do not support the growth of mouse colony-forming cells, it was possible to rescue a low number of human progenitor cells for up to 8 weeks posttransplantation (91). The BNX model has been refined by the injection of human stromal cells engineered to produce human IL-3 along with human bone marrow cells (92). Human hematopoietic progenitors were recovered from the spleens and bone marrow of recipient mice for up to 9 months after transplantation. The coinjection of engineered human stromal cells improved the level of human cells in the bone marrow to approximately 6% (92).

The ability of a single cell to give rise to both lymphoid and myeloid progeny is a unique property associated with HSC. This property has been demonstrated in the BNX mouse model by transplanting human CD34+ umbilical cord blood cells transduced with a retrovirus containing a neomycin resistance (neo^r) gene (93). Following transplantation, a small number of human hematopoietic progenitor cells containing the neo^r provirus in the recipient animals were detected. If a myeloid progenitor cell and a T-lymphocyte containing the neo^r provirus are derived from the same progenitor cell, the provirus should be integrated into exactly the same genomic site in each type of cell. DNA was extracted from human myeloid colonies grown in semisolid medium and from individual human T cells. The insertion site of the provirus was demonstrated using inverse PCR. Inverse PCR amplifies circular fragments generated by digestion of DNA with an enzyme that cuts once inside the provirus and at other random sites throughout the genome. Using primers specific to a single region of the provirus for PCR, the circular fragments can be amplified, generating a specific fragment for each proviral insertion that can be identified by its DNA sequence (94). Many insertion sites were detected in the myeloid colonies, 4 of which were shared by T-lymphocyte clones isolated from the same mouse. DNA sequence analysis demonstrated that the proviruses were integrated into the same spot in the ge-

nome. These studies provided the first and most definitive evidence that among the human cells that engraft into the BNX mouse are cells that have the properties associated with HSC (93).

Mice homozygous for the *scid* mutation lack functional B and T lymphocytes due to defects in V(D)J recombination and double-stranded, break repair due to mutations in the gene encoding the catalytic subunits of DNA-PK and DNA-PK_{CS} (95). Improved levels of engraftment and proliferation of human bone marrow cells transplanted into CB.17 scid/scid (SCID) mice. Human cells were detected in the bone marrow of transplanted CB.17 SCID mice at levels of 3% or higher for 8–10 weeks (96). Higher levels of human cells were detected in CB.17 SCID mice treated with human stem cell factor (SCF) and PIXY 321, a fusion molecule between human IL-3 and GM-CSF (96). Human myeloid and lymphoid cells were detected in the peripheral blood of transgenic SCID mice transplanted with human bone marrow cells for up to 24 weeks in a line of transgenic mice that expressed the human GM-CSF, IL-3, and SCF genes (97).

The meticulous work of Shultz and colleagues showed that the NOD strain of mice, which is susceptible to Non-Obese Diabetes, was NK-cell-deficient (98). When the *scid* mutation was crossed onto this strain, the resulting NOD-SCID mouse was more immune-deficient than any other strain carrying the scid mutation (98). A number of groups have demonstrated that NOD-SCID mice make superior hosts for engraftment of human hematopoietic cells (76). NOD-SCID carrying the knockout allele of β-2 microglobulin are even more immune-deficient and are further improved recipients of transplanted human cells (99).

NOD-SCID mice have been further engineered to provide the optimal microenvironment for human hematopoiesis. SCID mouse models in which pieces of human fetal thymus and fetal liver are implanted under the kidney capsule demonstrated the presence of human T-lymphocytes and myeloid cells are detected in the peripheral blood of transplanted SCID-hu animals for 6–12 months (100). Examination of the engrafted organs revealed the presence of multipotential myeloid and erythroid progenitor cells, and a full complement of differentiating human T-lymphocytes (100,101). Further refinement of the SCID-hu model implanted human fetal bone, thymus, and spleen fragments (abbreviated BTS) into SCID mice. The SCID-hu BTS mouse that can support human hematopoietic cells of all lineages for 36 weeks or more (102). The fetal bone fragments can be directly injected with purified hematopoietic cells that can then be analyzed for their capacity to repopulate and proliferate (102).

The demonstration of clonal hematopoiesis on the SCID models has not been as clear as in the BNX mouse model. For example, Josephson et al. examined multiple clones of CD19+ B-lymphocytes, and expanded populations of single myeloid cells obtained from NOD-SCID mice transplanted with foamy virus-transduced cells 6 weeks earlier. Less than 20% of the proviral insertions present in the B-lymphocyte clones were also represented in myeloid cells (103). It is clear that NOD-SCID mice allow the engraftment and proliferation of both HSC and more committed progenitor cells. Recent data analyzing NOD-SCID mice at 12 weeks posttransplantation suggest that the hematopoietic cells present at the later stages of transplantation are derived from the most primitive cells (104).

D. Phenotype of Human HSC

The enrichment of human HSC has used strategies that are similar to those used to enrich mouse HSC. Human hematopoietic cells expressing lineage markers have no ability to form colonies in vitro and are inactive in the LTCIC assay (105). All human hematopoietic colony-forming cells express the glycoprotein CD34. Human CD34+ cells isolated from bone marrow, mobilized peripheral blood, umbilical cord blood, or fetal liver are used for clinical transplantation, indicating that the CD34+ population contains HSC. Human HSC can be separated from colony-forming cells based on the presence of the CD38 antigen (106). Colony-forming cells express CD38 (Lin− CD34+ CD38+), while the more rare Lin− CD34+ CD38− cells are the only cell type capable of generating extended LTCIC in vitro (70).

As described above, human CD34+ cells engraft and proliferate in the BNX, SCID, and fetal sheep models (92,107–110). Recently, several lines of evidence have demonstrated the existence of human CD34− HSC. Differential Hoechst staining of human bone marrow cells reveals a substantial number of CD34− cells in the side population (111). (Lin− CD34− CD38−) bone marrow cells have been shown to engraft and proliferate in the fetal sheep (112) and NOD-SCID mouse models (113). These results indicate that at least some human HSC are CD34−, while others are CD34+. The difficulty in separating the repopulating CD34− CD38− cell from the large number of other CD34− cells may have prevented the injection of sufficient numbers of CD34− cells in previous studies.

IV. HEMATOPOIETIC STEM CELLS AS TARGETS FOR GENE THERAPY

A. Requirements for Effective Gene Therapy

The ability of the HSC to completely repopulate the entire hematopoietic system following transplantation makes the hematopoietic system a particularly attractive target for a permanent correction of inherited or acquired defects (8–10,(114). Integration of new genetic material into the genome of HSC would ensure a continuous supply of modified hematopoietic cells in the transplant recipient. In addition, the repopulation of the thymus with the progeny of transduced HSC has been shown to induce immune tolerence to the transgene product, which would allow the production of a new protein in the recipient without triggering an immune response, a frequent problem encountered in gene therapy (115). In contrast, gene transfer into progenitor cells, which do not have the ability

to repopulate or self-renew, would be transient and would require periodic gene transfer procedures to maintain a supply of corrected cells. Adeno-associated viruses have been shown to integrate into the genome of cells cultured in vitro, and transduction of HSC with recombinant AAV vectors has been attempted (116–118). At the present time, the frequency of recombinant AAV integration into the HSC genome has been too low to predict that AAV-mediated gene transfer to HSC would be an efficient gene transfer strategy. Similarly, adenovirus vectors, which do not integrate into the host cell genome, would not be effective for HSC gene therapy (119). Oncoretrovirus and lentivirus vectors integrate into the host cell genome, and have been developed specifically for gene transfer into HSC (120–122). The development of oncoretroviral and lentiviral vectors is discussed in other chapters; this chapter will focus on the use of oncoretrovirus and lentivirus vectors to transduce HSC.

Successful oncoretrovirus-or lentivirus-mediated gene transfer into hematopoietic stem cells requires several critical events (123–125). First, the virus particle must enter the target cell. The interactions between the target cell and the virus are mediated by the envelope protein of the virus and specific receptor molecules on the surface of the target cells (126,127), and the choice of virus envelope can have dramatic effects on the efficiency of gene transfer. After the viral genome has entered the target cell, the reverse transcriptase (*pol*) protein packaged with the virus converts the RNA genome into a double-stranded DNA molecule (124,125). This step is regulated by the availability of deoxynucleotide triphosphate molecules (dNTP) in the cytoplasm. Cells in the G_0 phase of the cell cycle (like HSC) typically have low levels of dNTPs, while cells in the G_1 phase of the cell cycle have increased levels of cytoplasmic dNTPs in preparation for DNA synthesis (128,129). The double-stranded DNA enters the nucleus where the *pol* protein-mediated integration into the host cell DNA (Fig. 3) (124,125). After integration, the transferred gene must be expressed at the appropriate levels in the appropriate cell type to correct the genetic defect (121). For some hematopoietic diseases, such as ADA deficiency, the expression of the ADA enzyme in hematopoietic cells of all lineages might have a beneficial effect (10). However T-lymphocytes must express the ADA gene at relatively high levels for gene therapy to be effective (130,131). For other diseases such as IL-2 receptor common chain (γc) deficiency (X-SCID), expression in lymphoid cells in essential (132), while expression of γc in myeloid cells would have no beneficial effect on the disease (133).

B. Oncoretrovirus-mediated Gene Transfer

The Moloney Murine Leukemia Viruses (MMuLVs) were originally studied as the causative agents of leukemia in mice (16,17). The MMuLV life cycle is very well understood (124,125), and despite the fact that replication competent MMuLVs can cause leukemia in mice and monkeys, MMuLVs were the first retroviruses to be adapted for gene therapy (122). Murine retroviruses are classified based on differences in their envelope proteins. The envelope protein of ecotropic retroviruses binds to a murine basic cationic amino acid transport protein, mCAT (134,135). The 3 amino acid sequence of this protein that serves as the virus binding site is not conserved in CAT from other mammals (136–138), restricting ecotropic retrovirus transduction to mouse cells. The envelope protein from amphotropic retroviruses binds to a phosphate transporter protein, Pit-2 (139–141). The binding site for the amphotropic envelope is conserved among the different mammalian Pit-2 molecules, giving amphotropic viruses a broad host range that includes human cells. Currently, most human gene therapy trials have involved amphotropic retroviruses (123).

Other retrovirus envelopes have been adapted for packaging recombinant retrovirus vectors. The envelope protein of the Gibbon Ape Leukemia Virus (GALV) binds to a distinct phosphate channel protein known as Pit-1 (142,143), which is approximately 60% identical to Pit-2 at the amino acid level (139,140). The Pit-1 amino acid sequence that binds the GALV envelope protein is conserved in most mammalian Pit-1 proteins with the exception of mouse Pit-1. Recombinant virus particles with a GALV envelope (144) have been used to improve gene transfer into mature lymphocytes (145,146). The 10A1 virus is a laboratory recombinant whose envelope protein binds to mouse and human Pit-1 and Pit-2 (147).

The Vesicular Stomatitis Virus type G (VSV-G) envelope recognizes a cell membrane phospholipid as a receptor, allowing VSV-G retrovirus particles to transduce almost all cell types regardless of species (148–150). In contrast to most other retroviruses, virus particles containing the VSV-G envelope can be agglutinated and concentrated to titers 2 orders of magnitude greater than can be obtained with other retroviruses (151–153). The envelope of the endogenous feline retrovirus RD114 recognizes a neutral amino acid transporter protein, and the binding site is conserved among larger mammals, but not mice (154). Recombinant retrovirus vectors with the RD114 envelope have been used to transduce high levels of CD34+ cells that engraft into NOD-SCID mice and fetal sheep (155,156).

A variety of recombinant MMuLV gene transfer vectors has been described in previous chapters, each of which has advantages that may be exploited for gene transfer applications [for review see (122)]. The LTRs from different murine retroviruses express downstream transgenes differently in different cell types. Each LTR has its own advantages in terms of the level of expression and the cells in which the highest levels have been achieved. In general, MMuLV LTRs are most active in lymphoid cells, while the Harvey Murine Sarcoma Virus (157), Myeloproliferative Sarcoma Virus (MPSV), and Mouse Stem Cell Virus (MSCV) LTRs promote relatively high levels of transgene mRNA in both myeloid and lymphoid cells (158–160).

It is well documented that transgene expression in hematopoietic cells can be silenced over time (161). A detailed analysis of active and silent proviruses derived from the same hematopoietic stem cell identified site-specific methylation within

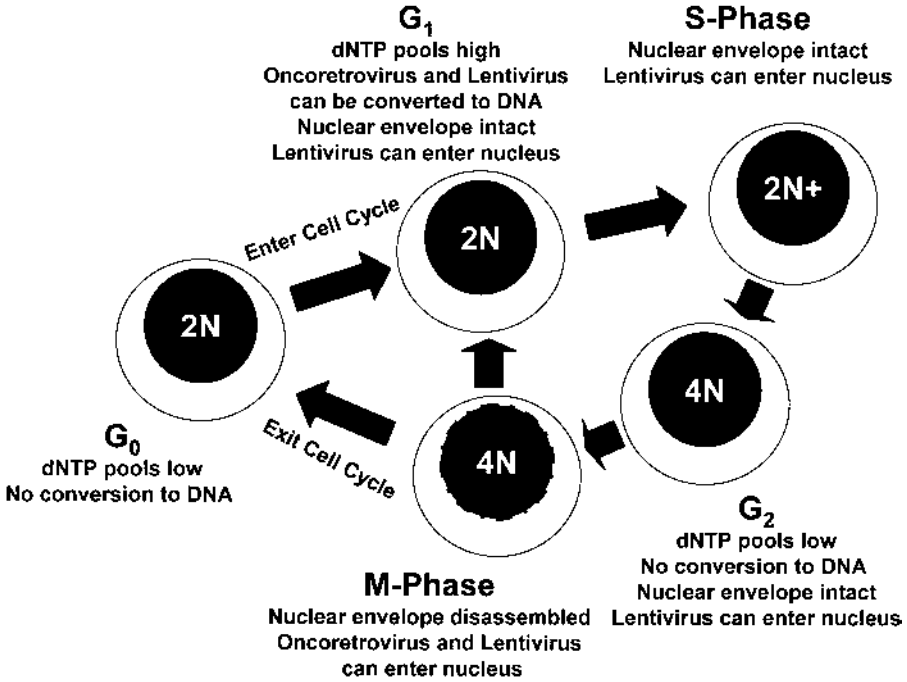

Figure 3 The relationship between the cell cycle and oncoretrovirus-and lentivirus-mediated gene transfer. RNA virus-mediated gene transfer is critically dependent on the abundance of virus receptors and the availability of deoxyribonucleotides for reverse transcription. The lentivirus life cycle differs from the oncoretrovirus life cycle in that lentivirus DNA genomes can be transported into the nucleus while oncoretrovirus integration requires the breakdown of the nuclear envelope during M phase.

the MMuLV LTR is associated with the silencing of gene expression (161). Mutation of the methylation sites combined with deletion of a negative regulatory region in the virus has dramatically improved the duration of gene expression in mouse hematopoietic cells (162,163). Gene expression from the MSCV LTR promoter has also been shown to be resistant to silencing (164).

C. Lentivirus-mediated Gene Transfer

After the RNA virus genome is converted to DNA, it must enter the nucleus for integration into the host cell DNA (123,124). This requirement limits oncoretrovirus-mediated gene transfer into HSC because oncoretrovirus DNA can only gain access to the genome when the nuclear envelope breaks down during mitosis. Since HSCs are usually quiescent or have a prolonged cell cycle (22,165–167), oncoretrovirus DNA must remain intact in the cytoplasm for a prolonged period of time. Lentiviruses, of which the Human Immunodeficiency Viruses HIV-1 and HIV-2 are the most familiar examples, have been shown to be able to integrate into the genome of nondividing cells (168). Similar results have been described with feline-derived lentivirus vectors (169,170). As described in previous chapters, the nuclear transport is mediated by two virion proteins, matrix and *Vpr*, which promote the transport of the HIV preintegration complex to the nucleus using the host cell nuclear transport machinery (171,172). The majority of lentivirus vectors are packaged with the VSV-G envelope (173), but recent studies have shown that lentivirus can also be packaged with amphotropic and RD114 envelopes (154,174). Compared to oncoretrovirus vectors, the lentivirus vectors integrated into irradiated or cell cycle arrested fibroblast cell lines at 5-to 20-fold greater efficiency (173,175). These studies have confirmed high rates of transgene integration into nondividing cells, including primitive human hematopoietic progenitor cells. However, direct comparisons in mouse models has shown that the efficiency of gene transfer into HSC is similar to that of oncoretrovirus vectors (176).

To date HIV vectors have been used only for gene transfer in animal models. The best application of the lentivirus technology has been the development of stable vectors containing the human β-globin gene and regulatory elements (177,178). For many years the inability of oncoretrovirus vectors to transfer an intact β-globin expression cassette had frustrated attempts to develop gene-based therapies for Sickle Cell Disease and the thalassemias (179). Several lines of evidence suggested that splicing of the β-globin retrovirus genomic RNA was responsible for the failure of an intact vector to integrate into the host cell genome. Lentivirus vectors are packaged in the presence of the *rev* protein, which suppresses splicing of

the lentiviral RNA, and containing the β-globin cassette were introduced intact into mouse HSC, with expression of therapeutic levels of β-globin (177,178). It is clear that there will be many safety concerns to address in the future in the design of HIV packaging and gene-transfer vectors. It appears that many of the modifications made to improve the safety of oncoretrovirus vectors could be used to improve the safety of lentivirus vectors.

V. MOUSE MODELS FOR RETROVIRUS-MEDIATED GENE TRANSFER INTO HSC

A. Proof of Principle

The initial attempts to introduce new genetic material into mouse HSC were made using recombinant ecotropic oncoretrovirus particles. The proof-of-principle experiments targeted a primitive hematopoietic progenitor cell, the Colony Forming Unit-Spleen (CFU-S). The CFU-Ss are derived from a single cell similar to the common Myeloid progenitor (CMP) (25,35), and gives rise to macroscopic colonies in the spleen of irradiated mice that contain mature cells of all myeloid lineages (25). The vectors used for these studies contained dominant selectable marker genes, either DHFR (180) or neomycin phosphotransferase (neor) (181,182). Mouse bone marrow cells were cultured on a monolayer of virus-producing cells, often in medium conditioned with the hematopoietic growth factor Interleukin-3 (IL-3) before recovery and injection into lethally irradiated mice. Proviral DNA was identified in high-molecular-weight DNA extracted from individual spleen colonies. These studies established that primitive progenitor cells had been transduced without destroying the ability of those cells to differentiate (180,181).

Extensions of these experiments examined lethally irradiated mice repopulated with cells that had been exposed to recombinant ecotropic oncoretroviruses. Southern blot analysis of proviral insertion sites in the bone marrow spleen and thymus revealed that a single HSC could repopulate all 3 organs (Fig. 4) (181,183). Cells containing the unique proviral insertion site persisted in the peripheral blood, marrow, spleen, and thymus of these mice for periods of 12 months or more, with no changes observed after 90 days (21,184–186). These studies established that a single HSC could be transduced without destroying the ability to repopulate the lymphoid and myeloid lineages.

In these early studies, less than 10% of recipient mice had long-term persistence of retrovirus-marked cells. In the rare positive mice, 10% or less of the hematopoietic cells contained a provirus (20,185). A variety of protocols were developed which improve the transduction of mouse HSC, all of which share common elements. The best results are obtained when the donor animals are treated with 5-fluorouracil, a cell-cycle-specific drug that is toxic to cycling cells like hematopoietic progenitor cells (16,187). The depletion of progenitor cells in the host may induce cycling of their more primitive precursors, which promotes retroviral integration (183). Combining IL-3 with IL-6 and SCF in the culture medium extended the viability of PHSC in culture without significant loss of repopulating ability, and improved gene transfer (185–189). Other cytokines, notably FLT3 ligand in combination with SCF, IL-6, and/or IL-3 (190,191), have had similar effects.

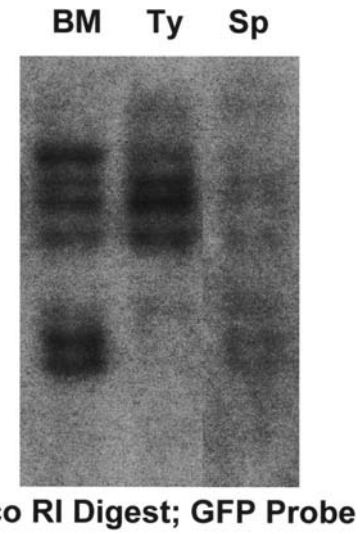

Figure 4 Transduction of mouse HSC. Mouse peripheral blood HSC were exposed to ecotropic retrovirus particles containing a GFP gene. Sixteen weeks after transplantation DNA was extracted from the bone marrow (BM), thymus (Ty), and spleen (Sp) and digested with Eco RI for Southern Blot analysis. Eco RI cuts once in the provirus, giving a unique insertion site for each proviral integration. Most of the bands are shared in DNA from the 3 hematopoietic organs, demonstrating the transduction of HSC.

Many protocols include 48-hour ''prestimulation'' of bone marrow cells in medium containing cytokines, prior to exposure to retrovirus particles (185,192). Recent experiments have shown that this prestimulation period appears to prevent the ''loading'' of retrovirus receptors with virus prior to the entry of HSC into cell cycle (166). Delaying the addition of virus until many HSC are competent for transduction optimizes the ability of the HSC to take up and integrate the provirus. Using the optimum combination of hematopoietic growth factors and transduction protocols, most laboratories achieve gene transfer in 100% of transplanted mice, and the proviruses are easily detected by Southern Blot analysis (Fig. 4).

B. Mouse Gene Therapy Models

Mouse models exist for many inherited diseases of the hematopoietic system. Gene therapy using retrovirus-mediated gene transfer into HSC followed by transplantation has been evaluated in several of these models. These include severe combined immune deficiencies (193,194), inborn errors of metabolism (195), and hemoglobinopathies (21,185,196)

genes. The first successful correction of a mouse model for human disease was reported by Wolfe et al. (197), who introduced the β-glucuronidase gene into the HSC of the mouse model for Sly Syndrome (β-glucuronidase deficiency). The authors reported prolonged life spans comparable to littermate controls and a reversal of the disease phenotype seen in untreated control animals. Subsequently, oncoretrovirus-mediated gene transfer has been used to correct both the X-linked (gp91phox deficiency) and autosomal (p47phox deficiency) forms of Chronic Granulomatous Disease (CGD) (198,199). Severe Combined Immune Deficiencies (SCID) caused by deficiencies of the Jak3 kinase and the common gamma chain (XSCID) have been cured by the transplantation of oncoretrovirus-transduced HSC (200–205). As noted above, lentivirus-mediated gene transfer of a human β-globin gene has significantly improved the phenotype of mouse models of β-thalassemia and Sickle Cell Disease (177,178).

The insertion of oncoretrovirus and lentivirus LTRs into the regulatory regions of specific genes is a well-described mechanism for leukemogenesis in animal models (16,17). Thousands of mice transplanted with oncoretrovirus-or lentivirus-transduced HSC have been evaluated in the disease models described above and related studies with other transgenes. Adverse events resulting in uncontrolled growth of hematopoietic cells have been described on two separate occasions. Bunting et al. described a myeloproliferative syndrome in mice transplanted with bone marrow cells transduced with an oncoretrovirus containing the p170 glycoprotein [or multiple drug resistance (mdr)] gene and expanded in vitro for 3 weeks. Follow-up studies demonstrated that the myeloproliferative syndrome was the result of the prolonged in vitro culture and the overexpression of the mdr gene (206). Li et al. described the insertion of a myeloproliferative syndrome in a single mouse caused by the insertion of a recombinant provirus near the Evi-1 locus (207). In strains of mice with high levels of endogenous wild-type oncoretrovirus, the development of hematopoietic malignancies is common, and oncoretrovirus insertions near the Evi-1 locus are found in a high percentage of myeloid leukemias in the AKXD-23 strain of mice (208). It was calculated that leukemia causing insertions represented less than 1/10,000 of all of the oncoretrovirus insertions in AKXD-23 mice (208). The AKXD-23 leukemias are similar to the one described by Li et al., demonstrating that recombinant oncoretrovirus insertions can cause leukemia, albeit at a low frequency (207). These findings stress the importance of extensive follow-up of animal models and the data and need to consider every possible means to ensure the safety of any gene therapy strategy.

VI. RETROVIRUS-MEDIATED GENE TRANSFER INTO HEMATOPOIETIC STEM CELLS FROM LARGER ANIMALS

A. Demonstration of Stem Cell Transduction

Most experiments using mouse models demonstrated that oncoretroviruses pseudotyped with the ecotropic envelope could introduce genes into HSC. Because ecotropic retroviruses can only transduce mouse cells, vectors with different pseudotypes were developed for gene transfer into HSC of large animals (123). The original large animal gene transfer protocols involved cocultivation of Rhesus monkey bone marrow cells on a layer of amphotropic oncovirus producer cells, similar to the successful strategies developed for mouse retrovirus-mediated gene transfer. Despite the use of similar protocols, the transduction of monkey HSC was more than 10-fold less frequent, with an average of less than 1% positive cells present in the best examples (209,210).

Safety concerns about the transduction of patient bone marrow cells with oncoretroviral vectors were increased after the discovery that replication-competent virus emerging from producer cells could cause leukemia in severely immunosuppressed Rhesus monkeys (211). These observations made it clear that only virus containing media shown to be negative for replication competent virus (and any other agents) could be used for patient studies. For most workers in the field, this meant transduction of hematopoietic cells in the absence of producer cells. One procedure seeded populations of Rhesus monkey CD34+ progenitor cells and HSC onto a previously established layer of bone marrow stromal cells that express SCF. The CD34+ cells were cultured for 96 hours in medium containing an amphotropic retrovirus containing the mouse ADA gene. The virus containing medium was replaced every 24 hours. After exposure to virus, the cells were collected and infused into the donor animal that had been irradiated while the CD34+ cells were in culture. The ADA provirus has been detected in 2% of mature blood cells of all lineages and bone marrow for a period of 9 years at the time of this writing (212,213). Mouse ADA was expressed at approximately 1–2% of the endogenous monkey ADA levels (212).

Another gene transfer protocol was evaluated in a dog bone marrow transplant model. Bone marrow cells from the donor animal were used to establish a long-term bone marrow culture (LTBMC). LTBMC require regular replacement of media, which offers the opportunity for repeated exposure to virus conditioned medium. At the end of 4 weeks in culture, the LTBMC was harvested and the cells used to transplant irradiated donor dogs. Gene transfer efficiency using this protocol was similar to that observed in monkey models with less than 1% of peripheral blood and bone marrow cells of the repopulated recipient containing the provirus (214–217). Transgene-marked hematopoietic cells were detected in the peripheral blood and bone marrow of these animals for periods of 5 years or more posttransplantation (217).

The discovery that the HSC in cytokine-mobilized peripheral blood cells or bone marrow cells from cytokine-treated animals were transduced at higher frequencies than untreated bone marrow HSC has led to significant improvements in gene transfer into Rhesus monkey and canine models. Using a combination of amphotropic or GALV pseudotyped oncoretrovirus particles, support on fibronectin, and cytokine primed peripheral blood or bone marrow cells, several groups have reported the transduction of 5–20% of HSC (218–220). Similar approaches have been attempted with lentivirus vectors,

but the efficiency of gene transfer has been similar to that of oncoretrovirus vectors (221).

VII. PROBLEMS AND DIRECTIONS FOR THE FUTURE

A. Low Titer

The low level of gene transfer into human and large animal HSC can be attributed to a combination of factors. One consistent problem with retrovirus-mediated gene transfer appears to be the relatively low titer of retrovirus preparations. The titer of even the best preparations of retrovirus rarely exceeds 1 million particles per milliliter of conditioned medium, and the movement of retrovirus particles in solution is limited by Brownian motion (222). Thus, most HSC do not come in contact with retrovirus particles under standard culture conditions. To increase interaction between retrovirus particles and HSC, investigators have used centrifugation to move HSC through a large volume of virus containing supernatant (223). Alternatively, the cells may be immobilized on a filter through which large volumes of retrovirus containing supernatant can be drawn (224,225). Both of these procedures have been shown to improve gene transfer into hematopoietic progenitor cells and may ultimately prove useful for the transduction of human HSC.

Another approach to increasing the contact between virus particles and HSC involves the use of fibronectin-coated gene transfer vesicles. It has been demonstrated that retrovirus particles and HSC bind specifically to a 35 kd fragment of fibronectin (226,227). The colocalization of the target cell and the retrovirus particle increases the probability that a cell will become transduced. The use of fibronectin has become standard in human gene transfer protocols. Titer can also be increased by concentration of virus particles in supernatant. A variety of protocols has been used to concentrate amphotropic, RD114, and VSV-G pseudotyped virus particles (228,229, 151–153).

B. Selection of Transduced HSC In Vivo

Recognizing the inefficient transduction of human HSC by oncoretroviral or lentiviral vectors, several groups have attempted to transfer drug resistance genes into HSC. These studies have a short-term goal of rendering bone marrow cells resistant to chemotherapeutic agents active against solid tumors, and a long-term goal of providing a means to select and amplify the rare transduced HSC. If the virus vector contained a therapeutic transgene along with the drug resistance gene, the result would be a high level of cells containing the therapeutic gene. This concept has been shown to be feasible in mouse models using the DHFR gene. When methotrexate selection is applied in the presence of nucleoside transport inhibitor nitrobenzylmercapt-purine riboside 5′ monophosphate (NBMPR-P), DHFR-expressing HSC demonstrate preferential survival (230). Similar results were observed when the human ENT-2 gene was introduced into mouse HSC followed by a similar selection protocol (231). Other strategies have involved the introduction of the MGMT gene followed by treatment of the animals with temozolomide, O6-benzylguanine, or similar drugs. As in the case of DHFR, transduced cells expressing MGMT were resistant to the effects of the drug treatment (232–234).

Alternatively, other groups have attempted to induce HSC self-renewal by expressing either chimeric receptor molecules that can be dimerized by small molecules, thus transducing a signal, or by expressing growth-factor receptors that ordinarily are not expressed by HSC. Treatment with either the small molecule or the cytokine triggers a ''growth switch'' that selectively amplified transduced HSC. This approach has been demonstrated in both mouse and nonhuman primate models (235–238).

C. Low Levels of Retrovirus Receptors

Many groups have observed that while the transduction of progenitor cells with amphotropic oncoretroviral vectors is relatively efficient, the transduction of HSC in the same population of cells is inefficient. These observations conflict with data in the mouse models with ecotropic oncoretrovirus particles, which can transduce both progenitors and HSC efficiently. One hypothesis proposed that ecotropic receptor levels were relatively high on mouse HSC, but that amphotropic retrovirus receptor levels were low or absent on mouse, primate, and human HSC.

Using a semiquantitative RT-PCR assay, Orlic et al. showed that the mRNA encoding the mouse ecotropic retrovirus receptor (mCAT) was easily detectable in mouse HSC, progenitor, and bone marrow cells. The mRNA encoding the amphotropic receptor (amphoR) was easily detectable in human or mouse hematopoietic cell lines and primary hematopoietic progenitor cells. However, amphoR mRNA was nearly undetectable in mRNA isolated from mouse and human HSC (239). Further investigation identified and isolated a rare subpopulation of mouse HSC that had higher levels of amphoR mRNA. To correlate receptor mRNA levels with transduction, amphoR positive and negative HSC were simultaneously transduced with genetically distinguishable oncoretroviruses, one packaged by ecotropic packaging cells, the other by amphotropic packaging cells. Greater than 90% of the animals in these experiments contained ecotropic proviral sequences in their hematopoietic cells, indicating that there was sufficient cell division to allow the integration of oncoretroviral vectors into HSC. More than 50% of mice repopulated with amphoR-positive HSC also contained amphotropic proviral sequences, while less than 10% of mice repopulated with amphoR-negative HSC contained amphotropic proviral sequences. In animals repopulated with amphoR-negative HSC, the relative number of ecotropic proviral sequences was 25-fold higher than the level of amphotropic proviral sequences, similar to the relative difference in the levels of ecoR and amphoR mRNA in HSC (239). Related work has demonstrated a low level of GALV receptor mRNA in human HSC as well (240). It would appear that a major obstacle to gene therapy for

human hematopoietic diseases is the low level of retrovirus receptor on the surface of HSC (Fig. 5).

The use of VSV-G pseudotyped retroviruses may eventually circumvent problems with low titer and low numbers of receptors. As noted previously, VSV-G particles can be efficiently concentrated to high titers without loss of activity (151,152). Since the VSV-G receptor is a membrane phosholipid (150), it would appear that there should be fewer problems with low receptor numbers. Problems associated with the toxicity of VSV-G *env* have slowed the development of VSV-G mediated gene therapy (149), but recent work with concentrated and purified VSV-G vectors has shown great promise in model systems (152,177,178). Other oncoretroviruses have been isolated from cats and sheep with novel envelopes that may recognize more abundant receptors on human HSC. For example, oncoretroviruses pseudotyped with the feline RD114 virus envelope have been shown to give high levels of gene transfer in canine models (241).

D. Quiescent HSC

Because Moloney Murine Leukemia Virus (MMuLV)-based retroviral vectors require cell division to integrate into the host cell genome, another obstacle to retrovirus-mediated gene transfer is the fact that most HSC are quiescent. Although relatively efficient transduction of mouse HSC can be achieved with ecotropic MMuLV vectors, it is not clear that human HSC are as prone to enter the cell cycle during the time they are exposed to the virus. One approach to this problem has been to expose human HSC to retroviral vectors under culture conditions that would lower the levels of cell cycle inhibitors. A recent study showed that the level of the cell cycle inhibitor p15(INK)4B could be decreased in human CD34+ progenitor cells by culture in serum-free medium containing antibodies against TGF-β. In addition, antisense oligonucleotides inhibited the expression of a second cell cycle inhibitor P27(KIP-1) in CD34+ cells and promoted cell cycling (242). Human CD34+ CD38− HSC were cultured in serum-free medium containing SCF, Il-6, IL-3, FL, anti TGF-β antibodies and antisense p27(KIP-1) oligonucleotides for 12 h and exposed to retrovirus vectors containing a neo gene for 12 h. Parallel cultures of cells were cultured in the same cytokines minus the anti TGF-β antibodies and antisense p27(KIP-1) oligonucleotides for 12 h and then exposed to the same virus. Cells from both conditions were equally capable of engrafting and proliferating in the BNX mouse model. One year after transplantation, no neo transgenes were observed in the human hematopoietic cells derived from cells not exposed to the anti TGF-β antibodies and antisense p27(KIP-1) oligonucleotides. In contrast the neo transgene was detected in about 10% of cells derived from CD34+ CD38− HSC exposed to anti TGF-β antibodies and antisense p27(KIP-1) oligonucleotides (242). These results clearly show that cell-cycle induction can promote retrovirus-mediated gene transfer into HSC without damaging the ability of the HSC to repopulate BNX mice.

The gene transfer problems relating to cell cycle may ultimately be overcome by the use of lentivirus-based vectors,

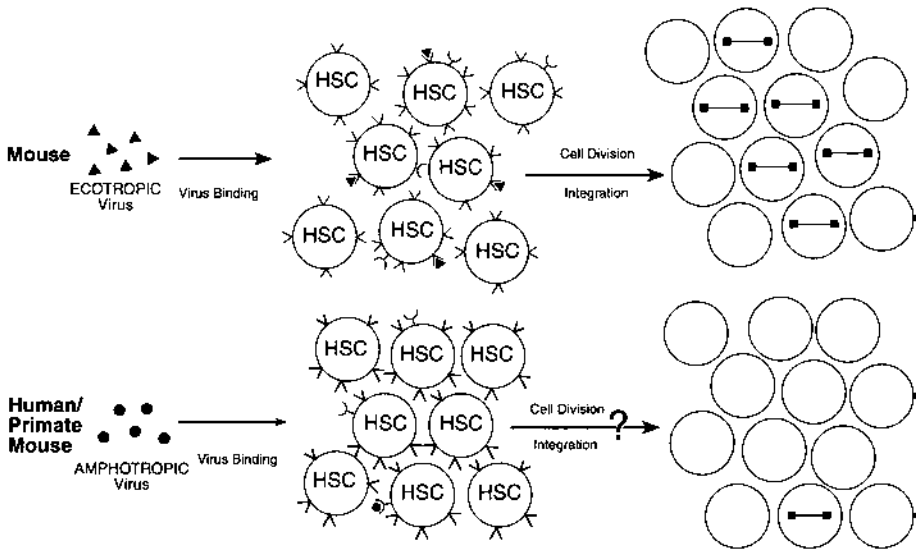

Figure 5 Comparison of transduction by ecotropic and amphotropic retrovirus particles. In the mouse (top), the high level of ecotropic retrovirus receptor allows many cells to bind a virus. Subsequent cell division allows these viruses to integrate into the genome. Amphotropic transduction of mouse primate or human HSC (bottom) is limited by low levels of amphotropic receptor expression on HSC. There are fewer opportunities for virus to bind to cells and therefore any cell division that occurs is unlikely to result in a viral integration.

which do not require cell division for integration. VSV-G pseudotyped lentivirus vectors have been shown to be greater than 20-fold more efficient at transducing freshly isolated human Lin− CD34+ CD38− HSC than MMuLV vectors (243). In addition, the expression level of the GFP reporter gene in lentivirus-transduced cells persisted for 5 weeks in culture, while expression of GFP from the MMuLV vector was lower and rapidly was silenced (243). Although the initial transductions of nonhuman primate and mouse HSC with lentivirus vectors have been similar to results with oncoretrovirus vectors, lentivirus mediated gene transfer still has theoretical advantages over oncoretrovirus-mediated gene transfer.

E. Summary

Improving the efficiency of gene transfer and the expression of the transferred genes are the critical basic research goals that will extend gene therapy from possibility to reality. Basic research into the biology of the PHSC has demonstrated low levels of retrovirus receptors and cell cycling as well as solutions to these problems. Basic research into retrovirus envelope proteins and backbones have lead to new recombinant retrovirus vectors that are capable of transferring and expressing genes at higher rates. As clinical experience is acquired in the use of retrovirus vectors for marking human HSC, it is clear that therapeutic gene transfer to human HSC could soon be a practical method for the treatment of ADA deficiency and other immune disorders. I predict that success in treating these diseases by gene transfer to HSC would be rapidly expanded to the treatment of a wide variety of other inherited and acquired hematological diseases.

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25

Gene Therapy for Hematopoietic Disorders

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I. INTRODUCTION

Clinical gene transfer to hematopoietic cells began on May 22, 1989 when autologous T-lymphocytes from a cancer patient were gene marked and transplanted back into the patient (1). Gene marking studies such as this have played an important role in the evolution of the field of gene therapy. Since 1989, over 250 patients have been enrolled in hematopoietic cell gene marking or gene therapy protocols. This chapter will review the practical issues involved in the conduct of a clinical gene therapy trial, as well as the published trials to date.

There are two general strategies to clinical gene transfer protocols for hematopoietic cells. The first strategy is to target the pleuripotential hematopoietic stem cell (HSC) with the goal of permanent gene transfer to all 3 hematopoietic cell lineages. Clinical HSC trials to date have involved transfer of both marker genes as well as therapeutic genes. The second strategy involves gene transfer into a single, terminally differentiated hematopoietic cell lineage. With this strategy, persistence of gene corrected cells is dependent on the lifespan of the target cell. At present, this approach has been applied clinically to allogeneic and autologous lymphocytes.

There are 3 broad categories of genes that have been used in clinical trials targeting hematopoietic cells: 1) marker genes, 2) suicide genes, 3) therapeutic genes. Marker genes provide a mechanism for detection of the transduced cells by introducing a gene product that is easily detectable by flow cytometry, enzymatic activity, or by conferring a drug resistance phenotype. Antibiotic resistance genes such as the bacterial neomycin phosphotransferase gene are most commonly used as markers. Suicide genes allow for in vivo elimination of transduced cells. The herpes thymidine kinase, for example, confers sensitivity of the transduced cell to the drug ganciclovir. There are a number of different types of therapeutic transgenes that have been utilized in clinical trials. Some are designed to interfere with the life cycle of the human immunodeficiency virus, some are designed to protect normal hematopoietic stem cells from chemotherapy, and others are designed to correct inherited monogenic disorders. Table 1 summarizes the published clinical gene transfer trials involving hematopoietic cells.

II. MURINE-BASED RETROVIRAL VECTORS

All clinical gene therapy trials to date targeting hematopoietic cells have utilized murine-based retroviral vectors from the oncovirinae subfamily. The simplicity of the murine retroviral genome has facilitated the development of a class of replication-incompetent vectors that can be produced by specially engineered producer cell lines. (Discussed in Chapter 1.) However, murine retroviral vectors can efficiently transfer genes only to actively replicating target cells (2). Relatively quiescent target cells such as HSCs, which reside predominantly in the G_0/G_1 phase of the cell cycle, are inefficiently transduced by murine retroviruses.

Most retroviral vectors that have been used clinically for hematopoietic cell gene transfer are based on the oncovirus called Moloney Murine Leukemia Virus (MoMLV) (3). Some clinical vectors are derived from the Harvey Murine Sarcoma Virus (HaMSV), and more recently vectors derived from the Murine Stem Cell Virus (MSCV) have been employed in clinical trials. HaMSV and MSCV are similar to MoMLV in overall structure and function, as are the modifications incorporated to make them replication-incompetent and safe to use in humans. The HaMSV and MSCV long terminal repeats (LTRs) may function

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Table 1 Published Clinical Trials of Hematopoietic Cell Gene Transfer

Transgene	Vector	Target Cell	Reference
Neomycin Resistance[a]	Retroviral	Hematopoietic Stem Cell	(33, 58, 59, 61, 64, 118)
$p47^{phox}$ (Chronic Granulomatous Disease)	Retroviral	Hematopoietic Stem Cell	(57)
Adenosine Deaminase (Severe Combined Immunodef.)	Retroviral	Hematopoietic Stem Cell	(34, 52, 92–94)
Multidrug Resistance 1	Retroviral	Hematopoietic Stem Cell	(86–90)
Glucocerebrosidase (Gaucher's Disease)	Retroviral	Hematopoietic Stem Cell	(38)
CD18 (Leukocyte adhesion deficiency)	Retroviral	Hematopoietic Stem Cell	(119)
Rev responsive element decoy	Retroviral	Hematopoietic Stem Cell	(120)
Neomycin Resistance[a]	Retroviral	Lymphocyte	(1, 113, 114, 121)
Adenosine Deaminase (Severe Combined Immunodef.)	Retroviral	Lymphocyte	(52, 53, 92, 93, 95)
Herpes Simplex Virus-Thymidine Kinase[b]	Retroviral	Lymphocyte	(115–117)
Transdominant Rev Protein (AIDS)	Retroviral	Lymphocyte	(120, 122, 123)

[a] Marker Gene Studies
[b] Suicide Gene Studies

better in hematopoietic stem cells and may be less subject to silencing, but this has not been proven in humans or nonhuman primates. Modifications to the MoMLV LTR have been made by some investigators to achieve a similar goal of preventing silencing related to methylation by altering methylation sensitive sites (4). The general description below of MoMLV also applies to the other related oncovirinae.

The MoMLV is composed of 2 copies of RNA ranging in size from 2–9 kb. In order to render these vectors replication-incompetent, the *gag* (core proteins), *pol* (reverse transcriptase and integrase), and *env* (envelope protein determining the host cell range or tropism of the retrovirus) genes are deleted. What remains are the 5′ and 3′ LTR sequences on each end of the construct along with the packaging (Ψ) site. The 5′ LTR functions as the promoter and enhancer region and the 3′ LTR contains the poly-A signal. The Ψ region serves as a binding site for the *gag* polyprotein, which packages the RNA into a viral core. The transgene is cloned into a site downstream of the Ψ region. In some vectors, splice donor and acceptor sites are retained or deliberately engineered into the Ψ region to generate, from a portion of the full-length mRNA, a subgenomic mRNA that more efficiently translates the downstream inserted cDNA open reading frame.

Replication-incompetent viral particles are produced in specifically engineered ''packaging cells.'' Packaging cell lines such as PA317 (5) (NIH3T3 derived murine fibroblasts), AM12 (6), Ψ crip (7), or 293SPA (8) (293 derived human embryonal kidney cells) constitutively express the *gag*, *pol*, and *env* proteins and therefore secrete empty virions into the culture media. When plasmid DNA of the retroviral vector is transfected into the packaging cells, clones of producer cells can be selected that secrete into the culture media replication-incompetent but infectious virions containing the transgene. The culture media, known as ''viral supernatant'' can then be collected and used ex vivo to infect the desired target cells.

An important issue regarding retrovirus vector transduction of hematopoietic cells is that stem cells and lymphocytes express relatively low levels of the surface receptor required for binding of the most widely used amphotropic envelope packaging element (9). Some studies have suggested that packaging lines using the envelope proteins of the Gibbon Ape Leukemia Virus (GALV) or the feline endogenous virus (RD114) may be advantageous for targeting both stem cells and T lymphocytes because these cells have higher levels of receptor for this envelope (10,11).

III. PRACTICAL CONSIDERATIONS OF HEMATOPOIETIC CELL GENE THERAPY

The following section outlines the 3 phases of a retroviral-based hematopoietic stem cell (HSC) and lymphocyte gene transfer protocol: 1) hematopoietic cell procurement, 2) ex-vivo gene transfer, and 3) reinfusion of the corrected cells. (Fig. 1)

A. Gene Transfer to Hematopoietic Stem Cells

1. Hematopoietic Stem Cell Procurement

Collection of large numbers of autologous hematopoietic stem cells is the first step in an HSC gene therapy protocol. The 3 sites from which HSC can be harvested are: 1) bone marrow, 2) cytokine or chemotherapy/cytokine-mobilized peripheral blood, and 3) umbilical cord blood. Although the precise phenotype of a true HSC is unknown, large numbers of HSCs are contained in a population of cells expressing the CD34 antigen (12). $CD34^+$ cells make up 0.5–5% of nucleated cells in the bone marrow and only a fraction of these are HSCs. HSCs can be safely aspirated from bone marrow of the poste-

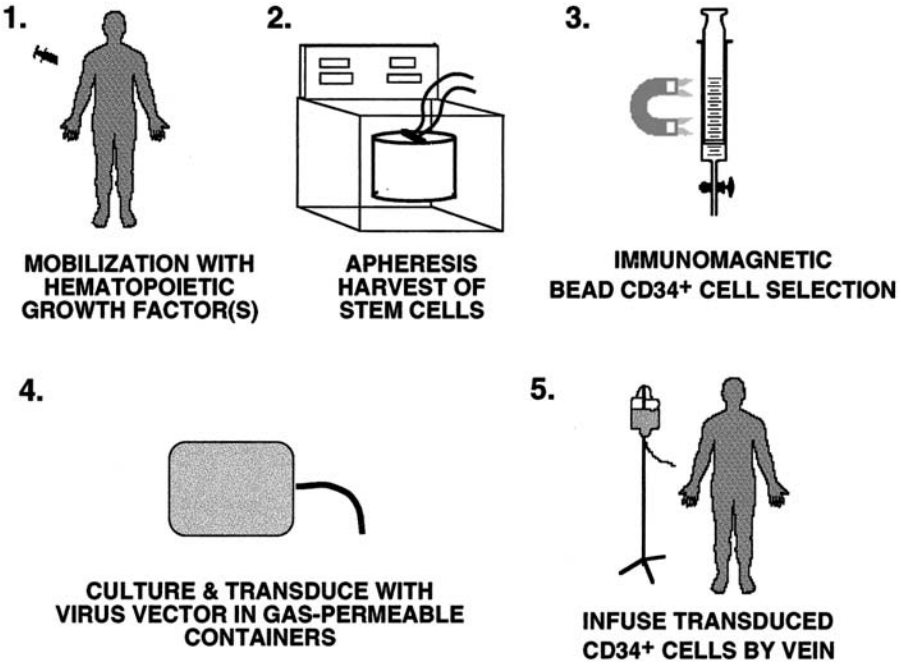

Figure 1 Clinical scale hematopoietic stem cell gene therapy scheme. See the color insert for a color version of this figure.

rior superior iliac crest in a minor operative procedure. The major side effects of this procedure include mild discomfort at the aspiration site and an occasional hematoma. For smaller individuals, symptomatic anemia may require a blood transfusion, which, if anticipated in advance, can be an autologous unit. The major disadvantage of large volume bone marrow aspiration is that it must be done under general anesthesia, which adds risk to the procedure. Because of these issues, repeated large-scale marrow harvests are not desirable.

In most individuals, administration of Granulocyte-Colony Stimulating Factor (G-CSF) at 10–16 μg/kg per day subcutaneously for 5 or 6 days results in a transient 20- to 100-fold increase in the frequency of $CD34^+$ hematopoietic progenitor and stem cells in the circulation (a phenomenon termed stem cell mobilization). Other growth such as Flt-3-ligand (Flt3-L) (13–15), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) (16–19), and Stem Cell Factor (SCF) (20,21), which are used alone or in combination, are also effective for stem cell mobilization. Large numbers of $CD34^+$ cells ($2–10 \times 10^6$ cells/kg patient weight) may be obtained from a single or repeated apheresis procedure following mobilization with growth factors. Furthermore, the mobilization process may be safely repeated at 4- to 8-week intervals. Unlike the procurement of bone marrow, no operative procedure is needed and the entire process can be done without a hospital admission.

Dunbar and coworkers (22) have demonstrated improved gene marking in nonhuman primates when peripheral blood HSC mobilized with G-CSF and SCF are compared to bone marrow HSC. The same group later showed superior in vivo gene marking of nonhuman primate HSC mobilized with G-CSF and SCF compared to HSC mobilized with G-CSF and Flt-3 ligand or G-CSF alone (23). High resolution cell cycle analysis of cytokine mobilized peripheral blood HSC has revealed that these cells are more likely to have entered cell cycle and express higher levels of the amphotropic receptor mRNA (24–26). While certainly easier to collect, human gene marking studies have yet to demonstrate unequivocally that cytokine-mobilized peripheral blood HSCs are better targets for retroviral transduction than bone marrow HSCs. However, the logistical advantages to obtaining peripheral blood HSCs dictate these as the preferred source for gene therapy and other transplant purposes except when the donor has a poor $CD34^+$ mobilization response to cytokine adminstration.

Umbilical cord blood contains a higher concentration of primitive hematopoietic progenitors than bone marrow (27). Recent data suggests that the HSCs from umbilical cord blood may also express higher levels of the amphotropic retrovirus receptor (28), which may result in more efficient transduction with amphotropic retroviral vectors. On average 20×106 $CD34^+$ cells can be collected from the placenta at the time of delivery, which is approximately 10-fold less than what can be collected from mobilized peripheral blood of an adult. Because of the low efficiency of HSC transduction with current techniques, clinical application for gene-corrected umbilical cord blood stem cells may only be practical in neonates.

2. Ex Vivo Gene Transfer

Optimization of ex-vivo retroviral transduction conditions for HSCs has proven to be a formidable task. It appears that 48

to 72 h of culture in growth factors is required for quiescent lineage negative $CD34^+$ cells to enter the cell cycle and thus become receptive targets for retroviral tranduction (29). However, studies have shown that prolonged ex vivo culture of HSCs results in loss of long-term repopulating ability as a consequence of lineage commitment and of an acquired defect in the ability of cycling cells to engraft (30,31). This loss of repopulating potential with ex vivo culture may be gradual and to some degree reversible. Takatoku and coworkers manipulated the ex vivo culture conditions and growth factor combinations such that HSCs from nonhuman primates were first induced into cell cycle by using a combination of active cytokines, thereby facilitating retroviral transduction. Then, before the cells were transplanted back into the animal, they were returned to a quiescent state by incubating the cells in media containing only stem cell factor. This method of ''resting'' stem cells prior to reinfusion resulted in improved engraftment of gene-marked cells (32).

Measurable gene transfer into HSCs in the clinical setting has been reported with ex vivo transduction periods ranging from 6 to 72 hours (33,34). While the report using a 6-hour regimen appeared to succeed in achieving measurable gene transfer without use of growth factors (33), most investigators have found that growth factors and an ex vivo culture period of 48–96 hours are required for optimum transduction. Growth factors are also essential to prevent apoptosis of HSCs during prolonged ex vivo culture.

Some investigators have used autologous bone marrow stromal cell layers in an attempt to enhance transduction with retrovirus vectors while preserving reconstitutive potential (35–38). Despite encouraging results in small animal models, there is no evidence that use of bone marrow stromal layers has resulted in better outcome in human clinical studies. Not only does the establishment of autologous stromal culture require a bone marrow aspiration, but this approach greatly increases the complexity and handling involved during clinical scale-up. Moreover, to achieve the highest level of transduction efficiency of HCSs grown on stromal layers, it is still essential to add growth factors to the culture.

Much current investigation is focused on determination of growth factor combinations used without stromal layers that can achieve highest transduction while maintaining reconstitutive potential of the transduced HSC. Flt3-L (39) and thrombopoietin (TPO) (39,40) have emerged as important agents to add to the ex vivo culture in relatively high concentrations (100–300 ng/ml) to achieve these dual goals. These growth factors work optimally in synergy with other growth factors. SCFs at more modest concentrations of 50–100 ng/ml appear to be important as well. Interleukin 3 (IL-3) and interleukin (IL-6) have also been widely used ex vivo in clinical trials to enhance cycling and may help to prevent apoptosis of HSC. However, use of concentrations of IL-3 higher than 30–50 ng/ml may be detrimental to preservation of cells with long-term engraftment potential. Other factors essential for maintenance and development of lymphoid progenitors from stem cells, such as IL-7, have not been used clinically, but may in the future prove to be a growth factor for the transduction of lymphocytes (41,42).

A number of techniques have been devised to encourage the interaction of hematopoietic progenitors with viral particles during the ex vivo transduction period. Most investigators have opted to transduce a cell population enriched for CD34 expression. $CD34^+$ cell enrichment enables an improved stem cell/viral particle ratio, thereby optimizing the multiplicity of infection while using less of the valuable clinical grade retroviral supernatant. A variety of stem cell selection devices that use monoclonal antibodies specific for the CD34 antigen have been employed in experimental clinical protocols [Reviewed in (43,44)]. These devices are able to select large numbers of $CD34^+$ cells from a bone marrow or mobilized peripheral blood apheresis graft at 50–80% efficiency yielding a product which consists of 60–90% $CD34^+$ cells.

For reasons that are not well defined, centrifugation of target cells during incubation in a retrovirus vector supernatant increases transduction efficiency, a technique that has been termed ''spinocculation'' (45). The g-forces employed to achieve the effect are as low as $1200 \times g$ for 20 min, making it unlikely that the effect is due to sedimentation of individual virus particles. Cocultivation of the target cells on a confluent layer of retrovirus producer cells has also been shown to enhance transduction. However, regulatory issues related to the safety of cocultivation of HSC with producer lines make this approach impractical for clinical application.

One of the more exciting techniques to be described is the finding that a specific proteolytic fragment of fibronectin facilitates stem cell–retroviral particle interaction when this peptide is used to coat the surface of the culture vessel (46). Fibronectin, a prominent component of the extracellular matrix of bone marrow stromal cells, has numerous hematopoietic cell-binding domains. Moritz and coworkers have demonstrated binding of both viral particles and hematopoietic target cells to a proteolytic fragment of fibronectin that contains the CS1 binding site (47). The CS1 binding site of fibronectin interacts with the VLA4 adhesion molecule found on hematopoietic stem cells (48). Thus, when HSCs are incubated with retroviral particles in the presence of this specific fibronectin fragment, transduction efficiency is improved.

The availability of a clinical grade recombinant human C-terminal fibronectin fragment (CH-296) has facilitated its use in several clinical gene therapy trials targeting HSC. Even with retrovirus vectors of modest titer, acceptable transduction of $CD34^+$ cells can be achieved. Of note is that with retrovirus vectors at titers $>5 \times 10^6$ infective particles per mL, the use of CH-296 fibronectin fragment coated culture vessels can achieve transduction of 50–70% of $CD34^+$ cells routinely at clinical scale. It is also possible that use of fibronectin fragment coating may help to preserve the long-term engraftment potential of cultured HSC (49).

3. Transplantation of Transduced HSC

Following transduction, the HSCs are infused into a peripheral vein of the patient. Within 24 h, the majority of the HSCs have homed to the bone marrow. Experience with stem cell

transplantation for treatment of hematological malignancies has shown that the bone marrow can be completely reconstituted by transplanted HSCs (autologous or allogeneic) following myeloablative doses of chemotherapy and/or radiation. Because loss of long-term repopulating ability may occur during ex vivo transduction of HSC, it is unethical to rescue hematopoiesis in a myeloablated patient with ex vivo manipulated HSCs only. However, preclinical and clinical studies suggest that some degree of cytoreductive therapy administered prior to infusion is required to establish clinically relevant levels of gene marking. Using clinically applicable tools, long-term marking at levels of 5–10% can be achieved in nonhuman primates following the administration of high-dose total body irradiation (30,50,51). Long-term multilineage gene marking has not been demonstrated in large animals or humans without bone marrow conditioning. Aiuti and colleages were the first to employ nonmyeloablative bone marrow conditioning in a human gene therapy study for children with adenosine deaminase-deficient severe combined immunodeficiency (discussed in more detail below) (52).

B. Gene Transfer to Lymphocytes

1. Lymphocyte Procurement

With few exceptions, large numbers of lymphocytes circulate in the peripheral blood and are therefore easily harvested from gene therapy candidates using apheresis. Lymphocytes may also be harvested from special sites such as tumors. These cells are of particular interest since they may possess unique antitumor properties.

2. Ex Vivo Gene Transfer and Reinfusion

Compared to HSC, fewer hurdles exist in the quest to optimize retroviral gene transfer of lymphocytes. Since these cells are terminally differentiated, loss of phenotype during ex vivo manipulation is not a concern. T-lymphocytes, which are the most common target for lymphocyte-based gene therapy, are expanded in culture with agents such as IL-2 and/or monoclonal antibodies to CD3. While being cultured, many of the cells are stimulated into active phase of the cell cycle and become susceptible to permanent retroviral integration. It has been observed that with long-term culture of T lymphocytes, enrichment of CD8 T lymphocytes relative to CD4 T lymphocytes develops. This issue may be addressed by altering the ratio of cells added to the initial culture mixture (53).

Techniques that have been shown to improve lymphocyte transduction efficiency include: 1) the use of retroviral vectors pseudotyped with the Gibbon Ape Leukemia Virus (GALV) envelope protein, 2) upregulation of amphotropic or GALV retroviral receptor expression by growth in phosphate depleted media, and 3) transduction in a culture vessel coated with the CS1 fibronectin fragment (54,55). Incorporation of these techniques together in the same protocol can yield transduction efficiency of 50–60%.

Reinfusion of the gene-corrected lymphocytes takes place as would any routine infusion of cell products. Cytoreductive conditioning of the recipient appears not to be necessary to achieve persistence of the transplanted lymphocytes, particularly where the therapeutic gene may provide a survival advantage (i.e., correction of ADA SCID).

C. Clinical Scale-up

The transition from a laboratory-based gene transfer assay to one that is ready for inclusion in a clinical protocol can be quite challenging. The most obvious differences relate to the number of hematopoietic progenitors that must be transduced at one time. Large volumes of retroviral supernatant must be produced in a facility licensed to provide clinical grade material. It is common to find a decrement in the viral titer of the clinical material compared to what is obtained in the laboratory. Besides the requisite sterility and endotoxin testing, the product must always be tested for the presence of replication-competent retrovirus. Regulatory and proprietary issues regarding use of reagents or devices often hinder the ability to replicate in the clinical setting what is done with ease in the laboratory. Performing retroviral transduction in a clinically approved facility may require modifications of a laboratory optimized assay (56). Use of closed-system, gas-permeable, culture containers compatible with the standard sterile transfer techniques used by blood banks is one method that has been adapted for this purpose (57).

IV. HUMAN GENE TRANSFER TRIALS

A. Hematopoietic Stem Cell Gene Transfer Studies

1. Marker Gene Studies

The insertion of marker genes into hematopoietic stem cells has been useful in the evolution of gene transfer technology and has led to a better understanding of autologous stem cell transplantation. The first, and arguably the most successful of these studies was reported by Brenner et al. in 1993 (33). This study enrolled 20 patients under the age of 20 who were candidates for autologous bone marrow transplantation for acute myelogenous leukemia or neuroblastoma. Bone marrow was harvested as the patient recovered from a cycle of cytotoxic chemotherapy. Two-thirds of the harvest was immediately frozen and the remaining one-third was transduced with a retroviral vector containing the neomycin resistance gene. In vitro transduction efficiency, measured as percentage of hematopoietic colonies resistant to G418 (neomycin analogue), ranged from 2–14%. The transduced and unmanipulated bone marrow cells were infused after the administration of high-dose chemotherapy. Using PCR analysis, the marker gene was detected in approximately 5% of bone marrow mononuclear cells 1 month following transplantation. G418 resistance was observed in 5–20% of bone marrow colony forming units (CFU) in 5 of 5 evaluable patients at 1 year, and in 2 patients at 18 months. Three important principals emerged from this study. First, the study proved that long-term repopulating cells could be successfully gene marked ex

vivo. Second, the study demonstrated that autologous marrow infusion following high-dose chemotherapy participates in the marrow recovery. Finally, the authors also reported that tumor cells obtained from patients who relapsed after the gene-marked, autologous BMT contained the neomycin resistance gene, suggesting tumor cell contamination of the autologous stem cell graft (58). Deisseroth et al. performed a similar gene-marked, autologous, BMT study in patients with chronic myelogenous leukemia where gene-marking of normal bone marrow CFU was demonstrated 6 months postinfusion. The investigators also demonstrated gene-marked tumor cells at relapse, suggesting tumor contamination of the autograft (59). Two other studies were unsuccessful at demonstrating tumor contamination of autografts, which may be a consequence of much lower rates of ex vivo gene transfer (60,61).

Dunbar et al. employed HSC transduction conditions optimized by others with animal models (36,62,63) in an attempt to improve the low ex vivo transduction efficiency reported by Brenner et al (33,58). This gene-marking study also set out to compare the engraftment capabilities of bone marrow and peripheral blood stem cells (PBSC) and their suitability as targets for retroviral transduction (64). Bone marrow and chemotherapy/cytokine-mobilized peripheral blood mononuclear cells were procured from breast cancer and multiple myeloma patients who were candidates for autologous stem cell transplantation. Two-thirds of each product was cryopreserved without manipulation. $CD34^+$ cells were purified from the remaining one-third of the mobilized peripheral blood, and bone marrow product and retroviral transduction of each product was performed using 1 of 2 molecularly distinct retroviral vectors containing the neomycin resistance gene. Since the bone marrow and peripheral blood $CD34^+$ cells were not transduced with the same vector, the contribution of each to engraftment following autologous stem cell transplantation could be tracked using PCR. Ex vivo transduction conditions consisted of a 72-hour culture of the target cells with retroviral supernatant supplemented with the hematopoietic cytokines IL-3, SCF, and IL-6 (IL-6 omitted from the culture of $CD34^+$ cells from multiple myeloma patients). All transduced and unmanipulated cells were pooled and reinfused following high-dose chemotherapy administration. The ex vivo transduction efficiency as measured by a clonogenic assay was 18–24%. Following transplantation, gene-marked bone marrow and peripheral blood cells were detected in 10 of 10 evaluable patients at a frequency of 0.1–1% using a semiquantitative PCR technique. At 600 days post infusion, only 1 patient had detectable levels of gene-marked cells. The authors did not identify any significant difference between bone marrow or peripheral blood stem cells as targets for retroviral gene transduction.

2. Chronic Granulomatous Disease (CGD)

CGD is an inherited immune deficiency caused by genetic mutations in any of the 4 subunits of the phagocyte NADPH oxidase ($p47^{phox}$, $p67^{phox}$, $gp91^{phox}$, $p22^{phox}$) resulting in the inability of phagocytes to produce microbicidal superoxide and hydrogen peroxide. CGD patients are prone to recurrent bacterial and fungal infections as well as granuloma formation (65). Stem cell transplantation can cure CGD, indicating that stem cells are the appropriate target for gene therapy (66–70). Preclinical work demonstrated that normal oxidase-positive neutrophils differentiate from CGD stem cells transduced with retroviral vectors encoding the corrective normal oxidase subunit cDNA (71–73). Based on this work, investigators at the National Institutes of Health have undertaken two clinical gene transfer studies to treat CGD.

The first study targeted CGD patients with deficiency of the $p47^{phox}$ subunit (57). The $p47^{phox}$ cDNA was cloned into the MoMLV-based MFGS retrovirus vector, and this vector was packaged by the murine Ψ-crip amphotropic packaging cell line. Mobilized PBSC were apheresed from patients after treatment with G-CSF followed by $CD34^+$ enrichment using an immuno-paramagnetic bead-selection device (Nexell Isolex®). Retroviral transduction of the PBSC was performed in serum-free media for 6 h in the presence of hematopoietic cytokines (PIXY321, G-CSF) for 3 consecutive days. The transduced PBSC were then transfused into the patient without marrow conditioning. Ex vivo transduction efficiency was determined by several methods, including quantitative assessment of oxidase-positive (Nitroblue tetrazolium dye test) myeloid colonies as well as determination of vector copy number using Southern blot hybridization. The percent of oxidase positive colonies ranged from 9 to 29%. This was closely correlated with the vector copy number of 5 to 18% found in transduced and cultured PBSC. Following intravenous infusion of transduced autologous $CD34^+$ cells into patients, the appearance of oxidase-normal neutrophils in the peripheral blood was assessed using a highly sensitive flow cytometry assay based on the increased fluorescence of oxidized dihydrorhodamine 123 (DHR) (74). Oxidase-normal neutrophils first appeared 2 weeks after transplantation and peak correction occurred after 3–6 weeks. The frequency of corrected neutrophils ranged from 0.004 to 0.05%.

The NIH investigators then performed a follow-up study that targeted CGD patients with the X-linked form of CGD caused by mutations in the transmembrane $gp91^{phox}$ subunit (personal communication). All 5 patients enrolled in this trial had the $gp91^{phox}$ null form of X-linked CGD and had previously received allogeneic normal granulocytes as treatment for severe infection. A high titer MFGS-$gp91^{phox}$ retroviral vector was prepared using the human-derived 293 (8) amphotropic envelope packaging cell line. In this study, GM-CSF combined with Flt-3 ligand (Immunex Corp.) was used to mobilize $CD34^+$ cells for apheresis. This novel cytokine combination was used to improve mobilization because previous studies had shown that CGD patients have a moderate defect in the mobilization response to G-CSF (75). Immune-selected cells (Isolex 300I®) were exposed to retroviral supernatant in experimental gas-permeable containers, which were coated with a clinical grade preparation of the CS-1 fibronectin fragment, Retronectin™ (Takara Shuzo). Transduction occurred on 4 consecutive days in serum-free media supplemented with Flt-3 ligand, Pixykine 321(Immunex Corp.), and stem cell factor. As in the first trial, cells were then reinfused into the

patient without marrow conditioning. Two or 3 cycles of gene therapy were administered at approximately 50-day intervals. Ex vivo transduction efficiency was assessed by flow cytometry using an anti-gp91 monoclonal antibody as well as by nitroblue tetrazolium dye testing for oxidase-positive colonies. 75–85% of $CD34^+$ $CD38^-$, Lin^-, HLA DR^- cells were positive for the gp91 protein as detected by flow cytometry after 7 days of in vitro culture. 50% of the myeloid CFU were found to be oxidase positive. Gene-corrected neutrophils were detected in 3 of the 5 patients beginning 2–3 weeks following the infusion of cells. One patient peaked at almost 0.15% oxidase-positive neutrophils. Similar peaks of corrected neutrophils were seen after each cycle, but the size of the peak correlated with the dose of gene-corrected cells that was infused. Gene-corrected cells gradually disappeared from the peripheral circulation approximately 3 months after the last cycle. In 1 patient, corrected neutrophils were recovered from the pus of a liver abscess that predated the time of enrollment in the study. The inability to detect corrected neutrophils in 2 of 5 patients may be a result of immune-mediated elimination of gene-corrected cells in this protein-null, inherited disorder.

3. Multidrug Resistance (MDR1) Gene Transfer

The MDR1 gene encodes a drug efflux pump called P-glycoprotein [Reviewed in Refs. (76–78)]. This pump confers resistance to a variety of anticancer agents including doxorubicin, mitoxantrone, vincristine, etoposide, and taxol. MDR1 can be used as both a therapeutic gene and as a selectable marker for other therapeutic genes (79).

In most cases, myelosuppression is the dose-limiting toxicity of chemotherapeutic agents used to treat cancer. Therefore, if the transfer of MDR1 into HSC is able to attenuate myelosuppression, higher and potentially more effective doses of chemotherapy could be delivered safely to the patient. Proof of this principal was provided in transgenic mice engineered to constitutively express MDR1 (80). These mice could tolerate several-fold higher doses of taxol and daunomycin than their wild-type counterpart (81,82). A similar effect has been demonstrated using retroviral gene transfer of MDR1 into HSC (83–85).

Results from the early studies were severely compromised by low levels of ex vivo transduction of HSCs, a low transplanted dose of transduced HSCs, and by the advanced stage of the cancer patients that were treated (86,87). Subsequent studies were notable for the fact that they provided evidence that in a few select patients, engrafted MDR1 transduced HSCs could reduce the intensity of the neutrophil nadir following high-dose chemotherapy and that repeated cycles of chemotherapy provided them with a selective advantage (88–90). The most notable MDR1 clinical trial came from Abonour and coworkers (89). The transgene vector consisted of a Harvey murine sarcoma virus backbone packaged in the AM12 amphotropic packaging cell line. Autologous peripheral blood $CD34^+$ cells were transduced on dishes coated with the fibronectin derivative CH-296 in media containing fetal calf serum and cytokines (SCF and IL6 or G-CSF/megakaryocyte growth and development factor[MGDF]/SCF) and reinfused into the patient following conditioning with VP16 and carboplatin. Peripheral blood cells containing the transgenes were detected by quantitative real-time PCR in 7 of 12 patients, with 1 patient as high as 5.6% 2 weeks following the transplant. No definitive evidence of hematopoietic chemoprotection was demonstrated when the gene therapy patients were treated with further chemotherapy. However, there was an increase in the percentage of transgene-containing cells in 6 patients following additional chemotherapy, suggesting a selective advantage for these cells. Importantly, no unexpected adverse events occurred in the study participants.

Safety issues regarding the use of MDR1 as a selectable marker have been brought into question by a recent study that demonstrates development of a myeloproliferative syndrome in mice transplanted with MDR1-transduced cells. Of note is that the transplanted cells had been subjected to prolonged ex vivo culture (91). Further studies in large animal models are needed to confirm this possible toxicity. Use of MDR1 and other promising selectable markers such as mutant dihydrofolate reductase is discussed in further detail in Chapter 21.

4. Adenosine Deaminase Deficiency (ADA)

The inherited deficiency of adenosine deaminase (ADA) is responsible for approximately one-quarter of the cases of severe combined immunodeficiency (SCID). In the absence of ADA, lymphocytes accumulate high levels of 2′-deoxyadenosine, which is converted to the toxic compound deoxyadenosine triphosphate. The result is a patient with profound T and B lymphocyte dysfunction that is susceptible to infections with opportunistic pathogens. ADA deficiency is an attractive target for gene therapy because corrected lymphocytes have a survival advantage over noncorrected, ADA-negative lymphocytes. Multiple gene therapy trials for ADA SCID targeting HSC as well as lymphocytes have been performed (34,52,53,92–95). Discussion in this section will focus on HSCs as the target for gene therapy.

Van Beusechem and coworkers laid the groundwork for HSC-based, ADA gene transfer by demonstrating in nonhuman primates that prolonged low-level expression of human ADA from peripheral blood cells was possible after bone marrow cells were transduced with retrovirus containing the ADA cDNA (96).

In 1995, Bordignon and coworkers in Milan, Italy published results of a clinical trial in which both peripheral blood lymphocytes and HSCs were transduced with 2 molecularly distinct retroviral vectors carrying the ADA and neomycin resistance genes and then reinfused into the patient without cytoreductive preconditioning (92). One patient received 9 injections of gene-corrected cells over a 2-year period. The other patient received 5 injections over 10 months. Prolonged detection of both transgenes were observed in both bone marrow and peripheral blood myeloid and lymphoid cells for the entire 35-month period of follow-up. Until the 35-month time point, the vector used for HSC correction was detected only in circulating granulocytes and bone marrow cells but not in

the lymphocytes. Gene-marked lymphocytes contained only the vector used to transduce lymphocytes. At 35 months however, lymphocytes derived from the gene-corrected HSCs were first detected and subsequently became more prevalent than those that carried the lymphocyte-specific vector. For ethical reasons, all patients treated in this study were treated concomitantly with PEG-ADA, thereby blunting the inherent selective advantage that exists for the gene-corrected cells. In 1 patient however, immune function became progressively dysfunctional despite PEG-ADA therapy and sustained long-term expression of the ADA gene in genetically modified lymphocytes. This provided the investigators with the rational for discontinuation of PEG-ADA therapy, with hope of restoring a selective advantage to the corrected cells and improving immune function (97). Prior to discontinuation of the PEG-ADA, the patient received additional gene-corrected autologous lymphocytes. The results of this intervention were positive. There was an increase in total peripheral blood T-cell numbers, an increase in intracellular adenosine deaminase levels, and normalization of in vitro T-cell responsiveness to anti-CD3 and alloantigens. Finally, the patient showed evidence of improved humoral immune function as measured by generation of anti-ψX174 antibodies following vaccination. However, the investigators also measured a concomitant increase in the toxic metabolites of adenosine in red blood cells, suggesting that despite the encouraging clinical effects of PEG-ADA withdrawal, repair of the underlying metabolic defect was incomplete.

Using improved transduction methodology and, for the first time in the setting of gene therapy for a nonmalignant disorder, low-intensity bone marrow conditioning (Busulfan 4mg/kg), the Italian group in collaboration with investigators from Israel transplanted 2 additional PEG-ADA SCID patients with gene-corrected HSC (52). Using this approach, the patients demonstrated an impressive increase in the number of peripheral blood T-cells within 2 months following gene therapy, obviating the need for PEG-ADA therapy and thus preserving the selective advantage of the corrected cells. Concurrent with increased T-cell numbers was normalization of T-cell proliferative responses, increased levels of IgM and IgA, and in 1 patient, a robust humoral response to vaccination with tetanus toxoid. The most notable finding from this trial is that 1 patient had sustained, high-level (5–15%) gene-marking in multilineage bone marrow and peripheral blood cells as measured by real time PCR. This finding will undoubtedly impact the development of future gene transfer trials in which the gene-corrected cells are not afforded a selective advantage, as they are in SCID.

Kohn et al. transduced $CD34^+$ cells isolated from umbilical cord blood of 3 ADA-deficient neonates with an MoMLV-based retroviral vector containing human ADA cDNA as well as the cDNA encoding the bacterial neomycin resistance gene (34). Cells were returned to the unconditioned recipient on the 4th day of life. Ex vivo transduction efficiency as measured by percent G418 resistant CFU ranged from 12–19%. Clonogenic myeloid precursors from the bone marrow were assayed at 1 year and found to be G418-resistant at a frequency of 4–6%. Concurrent with the infusion of gene-corrected HSC, the patients began ADA replacement therapy with PEG-ADA. As a result, the selective advantage of ADA positive clones was partially blunted. After 4 years of follow-up, the levels of gene-corrected cells in the peripheral blood mononuclear cell fraction has increased 50–100-fold in all 3 patients (93). The frequency of gene-corrected T-lymphocytes increased to 1–10%. This rate of increase was far greater than that observed in the granulocyte series (0.01–0.03%). In an attempt to assess clinical efficacy of gene transfer, PEG-ADA administration was discontinued in the patient with the highest level of gene-corrected T-lymphocytes. This resulted in reduction of S-adenosylhomocysteine hydrolase activity and loss of antigen specific T-lymphocyte reactivity, suggesting inadequate ADA production by the transduced cells. After 2 months, PEG-ADA replacement was restarted when oral monilia, sinusitis, and an upper respiratory tract infection developed. Despite the inability to demonstrate clinical benefit, the persistence of the transgene for over 4 years in both the lymphoid and the myeloid cell lines suggests that gene transfer into true long-term repopulating HSCs is possible from cells collected from umbilical cord blood.

5. X-linked Severe Combined Immunodeficiency

The majority of patients with SCID carry a mutation in the common γ chain of the receptors for interleukin 2, 4, 7, 9, and 15. Without a functional γ chain, there is complete absence of T-cell and NK cell development resulting in a profound immunodeficiency that is usually fatal in the first year of life. HLA-identical or more commonly, haploidentical parental bone marrow stem cell transplantation with or without antecedent cytotoxic bone marrow conditioning (due to selective advantage of normal T-cells) restores T-cell function and a relatively normal quality of life in 70–90% of patients. B-cell function is often deficient in patients who are transplanted without marrow conditioning. Such patients are dependent on life-long immunoglobulin supplementation to remain healthy.

The residual humoral immune defect, along with risks of graft vs. host disease and conditioning-related toxicity for patients treated with a traditional stem cell transplant led investigators from the Necker hospital in Paris to conduct the first stem cell gene transfer trial for X-linked SCID. $CD34^+$ bone marrow stem cells from 5 affected boys were tranduced with a Moloney murine leukemia virus (MFG) packaged in the Ψcrip producer line and reinfused without bone marrow conditioning. In 4 of 5 patients, normal functioning gene-corrected T-cells and NK cells were detected in peripheral circulation approximately 3 months after the transplant. As a result of the selective advantage for gene-corrected T-cells, total T-cell numbers reached normal levels within 6 months. Serum immunoglobulin levels normalized in 3 of 5 patients and was low but detectable in the 4th patient. All patients with successful engraftment of gene-corrected stem cell showed resolution of all stigmata of profound immunodeficiency, making this the first gene therapy trial to provide clear-cut clinical benefit. Gene-corrected cells were never detected in 1 of the patients

and he was subsequently successfully treated with allogeneic stem cell transplantation (98).

Unfortunately, excitement surrounding the success of this trial was tempered by the development of T-cell leukemia in 2 patients. Molecular analysis of the tumor cells revealed that malignant transformation most likely occurred as a consequence of insertional mutagenesis of LMO-2, a gene linked to T-cell leukemia (99,100). How this event will affect future gene therapy trials for X-linked SCID or other inherited stem cell disorders is not clear.

6. Fanconi Anemia

Fanconi anemia (FA) is an autosomal recessive disorder manifested by aplastic anemia, physical malformations, and cancer susceptibility (101). Eight separate genotypic groups of FA have been described (FA-A though FA-H) (102). Cells carrying the FA mutation are hypersensitive to DNA-damaging agents such as mitomycin C (103). Since stem cell transplantation successfully treats the hematological manifestations of FA (104,105), it is a logical candidate for stem cell gene therapy. In addition, there is evidence to suggest that normal stem cells may have a selective growth advantage over HSCs with the mutated FA gene (106).

After demonstrating in vitro that HSCs from patients with the FA group C (FAC) gene mutation could be functionally corrected by retroviral gene transfer of the normal FAC gene, Liu et al. initiated a clinical trial (107). Three children and 1 adult with the FAC mutation were treated with sequential cycles of autologous, G-CSF-mobilized CD34 selected progenitor cells that were transduced with a MoML V-based retroviral vector containing the cDNA for FAC and neomycin resistance. The cells were transduced in the presence of hematopoietic cytokines IL-3, IL-6, and SCF for 72 h and returned to the patient without cytotoxic bone marrow conditioning. The cell dose for patients 1 and 2 were extremely low due to poor CD34+ cell mobilization in response to G-CSF. FAC vector sequence was detected by PCR in the peripheral blood and bone marrow of all 3 patients at levels that ranged from 0.01–3%. Multilineage, peripheral blood-marking was detected in 1 patient for a 16-month period during which the patient received 4 cycles of gene transfer. The other patients had only transient bone marrow and peripheral blood positivity despite repeated cycles of gene therapy. Bone marrow sampling of each patient following the infusion of gene-corrected cells revealed an increase in the number of colonies resistant to the DNA-damaging effects of mitomycin C.

Radiation therapy delivered to patient 4 approximately 50 days following gene therapy for treatment of squamous cell carcinoma of the vulva resulted in detection, for the first time, of gene-marked cells in the peripheral blood. These gene-corrected cells were undetectable prior to radiation therapy, suggesting that they were afforded a selective advantage by the radiation treatments (108).

7. Gaucher's Disease

Gaucher's disease is an autosomal recessive disorder that results in a deficiency of the lysosomal enzyme glucocerebrosidase (109). This leads to the accumulation of glucosylcerebroside in macrophages throughout the reticular endothelial system. Although the clinical course is quite variable, most patients develop hepatosplenomegaly and painful, lytic, bone lesions. Conventional treatment includes stem cell transplantation or glucocerebrosidase supplementation.

Dunbar et al. has published the first clinical gene therapy trial for Gaucher's disease (38). Three patients received CD34-selected (Cellpro Ceprate) bone marrow or mobilized PB transduced on autologous stroma +/− cytokines with a retroviral vector containing the human glucocerebrosidase cDNA. Transduction efficiencies were low, ranging from 1–10% using a semiquantitative PCR assay. Transgene was detected in peripheral blood mononuclear cells (<0.02%) for 3 months in the patient whose cells were transduced with the highest efficiency.

8. Human Immunodeficiency Virus

HSC and mature T-lymphocytes are targets for anti-HIV gene therapy. Strategies include transfer of genes encoding ribozyme, which target the viral RNA genome, RNA decoys, and mutant transactivator genes that interfere with viral gene expression (110–112). These approaches, along with a review of the clinical trials are discussed in Chapter 31.

9. Summary

Techniques for gene transfer to HSC have advanced to the point where definitive clinical benefit has been achieved in a small number of patients with SCID. Sustained engraftment of transduced HSC whose progeny do not have a selective advantage is best demonstrated in the gene transfer studies where cytotoxic bone marrow conditioning was administered prior to the transplant (33,58,64,124). Besides reducing the number of resident naive stem cells, marrow conditioning results in a bone marrow microenvironment that is more conducive for engraftment of transduced HSC. Improvement in retroviral transduction conditions that utilize cytokines such as FLT-3 ligand and thrombopoietin have resulted in impressive rates of HSC gene transfer in myeloablated nonhuman primates (30,51). Alternative vectors such as those derived from lentivirus hold considerable promise for future HSC-gene-transfer strategies, assuming that their safety can be maximized. Given the newly described cases of insertional mutageneis that has complicated the X-SCID trial, risk vs. benefit of gene therapy will need to be reevaluated for each candidate stem cell disorder.

B. Lymphocyte Gene-transfer Studies

1. Gene-marking Studies

The first clinical gene-therapy trial involved gene transfer into lymphocytes and was undertaken in the late 1980s as a means of characterizing tumor-infiltrating lymphocytes (TIL) (1). TIL were isolated from tumors of patients with metastatic melanoma. These cells were expanded and then transduced with a retroviral vector containing the neomycin resistance gene. Following reinfusion, the marker gene was used to track

the migration of the marked lymphocytes. TIL were consistently found in the peripheral blood as well as tumor deposits for up to 2 months. A similar approach was employed to study the persistence of Epstein-Barr virus (EBV)-specific cytotoxic T-lymphocytes (CTL) generated ex vivo and infused as treatment of post bone marrow transplant EBV-related lymphoproliferation (113,114) EBV-specific CTLs marked with a neomycin-resistance vector were detectable in the peripheral circulation for 10 weeks post infusion.

2. Adenosine Deaminase (ADA) Deficiency

Blaese et al. were the first to use a therapeutic gene in a retroviral gene transfer trial for ADA-deficient SCID patients (53). Two patients with an incomplete response to PEG-ADA therapy were infused with T-lymphocytes transduced with an MoMLV-based retroviral vector containing the cDNA coding for ADA. The transduction efficiency of lymphocytes prior to infusion ranged from 0.1–10%. Patients were treated with multiple cycles of transduced T-cells over a period of 1–2 years. Gene-corrected cells were detected in circulation for 2 years following the final cycle of gene therapy, demonstrating a T-lymphocyte lifespan much longer than what was predicted. There was clear-cut evidence of improved cellular and humoral immune response following gene therapy in 1 of the 2 patients treated. This discrepancy in patient response was attributed to superior ex vivo transduction efficiency obtained in the responding patient. No toxicity was attributed to the conduct of the protocol.

Using the identical experimental design and retroviral vector, Onodera et al. accomplished similar results in 1 patient treated in Japan (95). These studies, in conjunction with the previously described Bordignon study (92) demonstrate the potential of lymphocyte-based gene therapy to provide prolonged clinical benefit.

3. Suicide Gene Transfer

The use of herpes simplex virus thymidine kinase (HSV-TK) gene and other ''suicide'' genes for gene therapy is discussed in Chapter 20. Introduction of the HSV-TK gene into a cell allows for the phosphorylation of nucleoside analogs such as ganciclovir. Once phosphorylated, ganciclovir becomes toxic to the cell as it is incorporated into DNA. There are 3 published hematopoietic cell gene therapy trials utilizing the HSV-TK. Lymphocytes were the target cells in both studies (115,116,117). In an attempt to make HIV therapy with autologous cytotoxic T-cells safer, Riddell et al. transduced HIV-specific cytotoxic T-cells with the HSV-TK gene (115). The transduced cells could then be eliminated with ganciclovir treatment if toxicity were to arise from their presence. The vector used to transduce the lymphocytes also carried the hygromycin phosphotransferase gene. Unexpectedly, the transduced lymphocytes were rejected by a brisk host cytotoxic T-lymphocyte response against the transduced cells and thus, efficacy could not be assessed.

In allogeneic bone marrow transplantation, lymphocytes taken from the bone marrow donor are often infused into the recipient to treat tumor relapse via the graft vs. tumor effect, or to aid in immune reconstitution. Significant toxicity may arise if transplanted lymphocytes mount an immunological attack against the recipient (graft vs. host disease). As in the Riddell study, Bonini et al. studied HSV-TK-transduced donor lymphocyte infusions as a method of protecting against graft vs. host disease (GVHD) (116). The vector used for transduction also carried the marker gene that coded for a truncated form of the human low-affinity receptor for nerve growth factor (NGFR) as well as the neomycin resistance gene. Gene-marked lymphocytes were detected in 7 of 8 patients available for analysis with a range of 0.01% to 13.4% of the total circulating lymphocytes. Three of the patients developed GVHD and were treated with ganciclovir. The percent of genetically modified lymphocytes decreased dramatically with complete resolution of GVHD in 2 of 3 patients. Partial resolution of GVHD occurred in the other. While there was no evidence of an immune-mediated elimination of the transduced cells, these bone marrow transplant patients were likely immunocompromised even more profoundly than the HIV+ patients studied by Riddell et al.

Using a similar strategy, Tiberghien and coworkers infused donor lymphocytes transduced with a MoMuL V-derived retroviral vector carrying the genes for HSV-TK and neomycin resistance into recipients of a T-cell-depleted allogeneic stem cell transplantation (117). Of the 12 patients treated, 4 developed clinically significant GVHD, prompting ganciclovir administration. Ganciclovir adminstration resulted in resolution of GVHD in 3 of 4 patients and, as in the earlier study, correlated with a drop in the number of gene-marked lymphocytes.

V. CONCLUSION

To date, all clinical trials involving transduction of hematopoietic cells have utilized murine-based retroviral vectors. When lymphocytes are the target cell, modest clinical benefit was observed in both the ADA and HSV-TK trials. This is attributable to the relatively high level of transduction efficiency using the latest techniques. Though clinical applicability of HSC gene transfer is much broader than for lymphocytes, long-term repopulating HSCs are less receptive to retroviral-based gene transfer. While alternate vectors such as lentivirus, and adeno-associated viral vectors are promising, a number of studies have proven that the retroviral vector can successfully target the long term repopulating cell, albeit with an extremely low efficiency. To improve the ability to transduce HSC, further knowledge of stem cell biology such as their cell cycle characteristics, their trigger for self-replication vs. lineage commitment, and the optimal ex vivo growth conditions will aid in the ability to transduce these cells. The production of high titer, replication-defective, retroviral supernatant is another technique that must be perfected. It is unclear to what extent host immune rejection of cells expressing the transgene will hinder progress of gene therapy for protein-null disorders or disorders where a heterologous gene product is produced.

And finally, it is becoming clear that some degree of cytoreductive bone marrow conditioning will be necessary to achieve clinically significant levels of HSC gene transfer.

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26

Gene Therapy for Cardiovascular Disease and Vascular Grafts

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The earliest, and perhaps most obvious, clinical embodiments of gene transfer technology have involved the treatment of rare genetic disorders. The development of effective methods of manipulating gene expression in vivo, however, coupled with an explosive growth in the understanding of changes in gene expression associated with the onset and progression of acquired diseases, has created a prospect for revolutionizing the clinician's approach to common disorders. As researchers learn more about the genetic blueprints of disease, they gain the potential to alter, or even reverse, pathobiology at its roots. Nowhere are these possibilities more avidly sought, nor more likely to impact a significant population of patients, than in the arena of cardiovascular disease.

Gene therapy has come to embrace both the introduction of functional genetic material into living cells as well as the sequence-specific blockade of certain active genes. These systems have included recombinant viral vectors that allow relatively efficient insertion of genetic information and oligonucleotides that can be used to alter native gene expression (1,2). This increased breadth of gene manipulation technology has accompanied the identification of genes that are either activated or repressed during disease. Recent discoveries have uncovered therapeutic targets (2,3) both for the improvement of conventional cardiovascular therapies, such as balloon angioplasty or bypass grafting, and for the development of entirely novel approaches, such as the induction of angiogenesis in ischemic tissues. As enthusiasm grows for these new experimental strategies, it is important for clinicians to be aware of their limitations as well as their strengths, and for careful processes of evaluation to pave the possible integration of these therapies into routine practice. This chapter will explore general principles of gene manipulation in the cardiovascular system, review a number of prominent examples of experimental reduction to practice, and is intended as a source to assist in the consideration of future developments in this exciting field.

I. GENETIC MANIPULATION OF CARDIOVASCULAR TISSUE

A. Gene Therapy Strategies

Gene therapy can be defined as any manipulation of gene activity, or gene ''expression,'' that influences disease. This manipulation is generally achieved via the introduction of foreign DNA into cells in a process known as transduction or transfection. Gene therapy can involve either the delivery of whole, active genes (gene transfer), or the blockade of native gene expression by the transfection of cells with short chains of nucleic acids known as oligonucleotides.

The gene transfer approach allows for replacement of a missing gene product, or for the ''overexpression'' of a native or foreign protein that can prevent or reverse a disease process. The transfer of a gene into a target cell leading to subsequent gene expression is known as *transduction*, and the new gene can be referred to as the *transgene*. Gene *replacement* or *aug-*

mentation involves the transfer of a gene that is either missing from a cell, present in a defective form, or simply underexpressed relative to the level of protein expression desired by the clinician. The protein expressed may be active only intracellularly, in which case a very high gene transfer efficiency may be necessary to alter the overall function of an organ or tissue. Alternatively, proteins secreted by target cells may act on other cells in a paracrine or endocrine manner, in which case delivery to a small subpopulation may yield a sufficient therapeutic result.

Gene blockade can be accomplished by transfection of cells with short chains of DNA known as antisense oligodeoxynucleotides (ODN) (4). This approach attempts to alter cellular function by the inhibition of specific gene expression. Genes are defined by a specific sequence of bases that make up the DNA chain. Antisense ODN are designed to have a base sequence that is complementary in terms of Watson-Crick binding to a segment of the target gene. They are generally 15–20 bases in length, which confers specificity to a single site within the genome. This complementary sequence allows the ODN to bind specifically to the corresponding segment of messenger RNA (mRNA) that is transcribed from the gene during expression. This binding of ODN to mRNA prevents the translation of RNA into the protein product of the gene (4).

Another form of gene blockade is the use of ''ribozymes,'' segments of RNA that can act like enzymes to destroy only specific sequences of target mRNA (5). Ribozymes contain both a catalytic region that can cleave other RNA molecules in a sequence-specific manner, and an adjacent sequence that confers the specificity of the target. Because the sequence recognition portion of the ribozymes is generally limited to approximately 6 bases, these gene-inhibitory agents are generally more susceptible to nonspecific interactions than their antisense counterparts.

A third type of gene inhibition involves the blockade of gene-regulatory proteins known as transcription factors. Transcription factors regulate gene expression by binding to chromosomal DNA at specific promoter regions, and this binding turns on, or ''activates,'' an adjacent gene. Double-stranded ODN can therefore be designed to mimic the chromosomal binding sites of these transcription factors and act as ''decoys,'' binding up the available transcription factor and preventing the subsequent activation of target genes (6).

The transfer of genetic sequences exogenous to the human genome has been envisioned, such as the gene encoding thymidine kinase (TK) from the herpes simplex virus. This has been used to enhance metabolic activation of the cytotoxic prodrug ganciclovir, which may be useful in treating vascular proliferative disorders such as restenosis (7). Scientists studying gene transfer technology have often relied on a class of genes known as *reporter* or *marker genes*. These genes encode proteins, such as a form of β-galactosidase found in *E. coli* (known as *lacZ* or *β-gal*) or a small fluorescent molecule known as green fluorescent protein (GFP), that can be detected easily via histochemistry, fluorescent microscopy, or other techniques that allow rapid identification and quantification of successful gene transfer.

B. Vectors for In Vivo Cardiovascular DNA Delivery

For the purpose of cardiovascular gene therapy, the ''ideal'' DNA delivery vector would be capable of safe and highly efficient delivery to all cell types, both proliferating and quiescent, with the opportunity to select either short-term or indefinite gene expression. This ideal vector would also have the flexibility to accommodate genes of all sizes, incorporate control of the temporal pattern and degree of gene expression, and to recognize specific cell types for tailored delivery or expression. While progress is being made on each of these fronts individually, researchers remain far from possessing a single vector with all of these characteristics. Instead, a spectrum of vectors has evolved, each of which may find a niche in different early clinical gene therapy strategies.

Viral vectors may represent nature's solution to the problem of efficient gene transfer; however, the attempt by humans to harness these resources has also been confounded by the biological barriers that have evolved to protect cells and organisms from viral infection. Immunological responses not only limit the efficacy of viral gene transfer, particularly when repeat administrations are considered, but the inflammatory response to viral antigens, even those associated with replication-deficient vectors, may impede or negate the benefits of expression of the transferred gene (8). Furthermore, engineering of viral genomes does not always preclude residual cytotoxicity in infected cells, and the possibility for regression to replication proficiency.

Scientists have therefore continued to explore nonviral avenues for achieving efficient DNA delivery. One advantage of nonviral delivery systems is that they can be used not only for gene transfer but also for the delivery of oligonucleotides and protein-nucleic acid complexes that can be used for alternative forms of genetic manipulation. Whereas *transduction* refers generally to the delivery of an intact gene to a target cell, and *infection* is used to describe the process of viral gene delivery, *transfection* is a term used to describe nonviral (i.e., physical/chemical) delivery of genes or oligonucleotides. Below is a brief description of DNA-delivery vectors that have been exploited in the cardiovascular system (Table 1).

1. Retroviral Vectors

Recombinant, replication-deficient retroviral vectors have been used extensively for gene transfer in cultured cardiovascular cells in vitro, where cell proliferation can be manipulated easily. Their use in vivo has been more limited due to low transduction efficiencies, particularly in the cardiovascular system where most cells remain quiescent. Nabel et al. (9) first demonstrated the feasibility of transducing blood vessels with foreign DNA in vivo by infecting porcine iliofemoral arteries with a recombinant retroviral vector containing the β-galactosidase gene. Several cell types in the vessel wall were transduced, including endothelial and vascular smooth muscle cells (VSMCs). Using a β-galactosidase retroviral vector to genetically modify endothelial cells in vitro, Wilson et al. (10) demonstrated expression up to 5 weeks after implanta-

Table 1 Comparison of Vectors Used for Cardiovascular Gene Transfer

	Efficiency (in vivo)	DNA integration	Duration of gene expression	Level of expression (max.)	Ease of preparation	Host response	Risks
Viral							
Retrovirus	+	Yes	Life-long	++	+++	+	Oncogenesis Viral mutation
Adenovirus	++++	No	1–2 weeks	++++	++	++++	Cytotoxicity Viral mutation
AAV	+	Sometimes	Life-long	+	+	+	Oncogenesis Viral mutation Viral contamination
Nonviral							
Liposomes	+	No	Limited	+	++++	+	Cytotoxic at high concentrations
Fusigenic Liposomes	++	No	Limited	+	+	+	Cytotoxic at high concentrations
Naked Plasmid	+	No	Limited	+	+++	+	Cytotoxic at high concentrations

+ = lowest, ++++ = highest, AAV = adeno-associated virus.

tion of a prosthetic vascular graft seeded with genetically transformed cells. The random integration of traditional retroviral vectors such as MMLV into chromosomal DNA involved a potential hazard of oncogene activation and neoplastic cell growth. While the risk may be exceedingly low, safety monitoring will be an important aspect of clinical trials using viral vectors. Recent improvements in packaging systems (particularly the development of ''pseudotyped'' retroviral vectors that incorporate vesicular stomatitis virus G-protein) have enhanced the stability of retroviral particles and facilitated their use in a wider spectrum of target cells.

2. Adenoviral Vectors

Recombinant adenoviruses have become the most widely used viral vectors for experimental in vivo gene transfer, and have been used extensively in animal models of cardiovascular disease (11). Adenoviruses can infect nondividing cells and generally do not integrate into the host genome. These vectors can therefore achieve relatively efficient gene transfer in quiescent vascular tissue, but transgenes are generally lost when cells are stimulated into rounds of cell division. Expression of DNA in a nonchromosomal, or episomal state, also appears to be less stable, and adenoviral transduction has proven to be transient in cells even in the absence of replication.

Many scientists have concluded that the immune response to adenoviral antigens represents the greatest limitation to their use in gene therapy. Conventional vectors have generally achieved gene expression for only 1–2 weeks after infection. It is not certain to what extent the destruction of infected cells contributes to the termination of transgene expression given that the suppression of episomal transgene promoters appears to occur as well. However, longer expression has been documented after injection of tissues in immune deficient mice. Even in the context of such reactions, the adenoviral vector has been postulated to provide an adjuvant effect that amplifies the immune response. In the vasculature, physical barriers such as the internal elastic lamina apparently limits infection to the endothelium, with gene transfer to the media and adventitia only occurring after injury has disrupted the vessel architecture. Although gene delivery to 30–60% of cells after balloon injury has been reported with adenoviral vectors carrying reporter genes, the fact that atherosclerotic disease has also been found to limit the efficiency of adenoviral transduction may pose a significant problem for the treatment of human disease.

3. Adeno-associated Viral Vectors

Adeno-associated virus (AAV) is a dependent human parvovirus that has not been linked to human disease (12). It can infect a wide range of target cells and can establish a latent infection by integration into the genome of the cell, thereby yielding stable gene transfer as in the case of retroviral vectors. Although AAV vectors transduce replicating cells at a more rapid rate, they possess the ability to infect nonreplicating cells both in vitro and in vivo. AAV is limited by its small size (transgenes cannot be longer than about 4 kb) and the need to eliminate helper viruses from viral preparations. The efficiency of AAV-mediated gene transfer to vascular cells, and the potential use of AAV vectors for in vivo vascular gene therapy, remains to be determined. However, a number of groups have reported successful transduction of myocardial cells after direct injection of AAV suspensions into the heart tissue, and these infections have yielded relatively stable

expression for greater than 60 days (13). It has not yet been clearly established whether long-term recombinant AAV transgenic expression is associated with genomic integration as is the wild-type AAV infection. In general, AAV transgene expression does not occur at significant levels during the first 2–4 weeks after infection, although the reason for this delay remains speculative.

4. Lipid-mediated Gene Transfer

Numerous nonviral methods are available for the delivery of DNA into cells in vitro, including calcium phosphate, electroporation, and particle bombardment. The development of similarly effective methods of in vivo transfection, however, has posed a significant challenge to cardiovascular and other clinical researchers who hope to avoid cumbersome and invasive steps of harvesting and culturing tissues or cells from the patient. The encapsulation of DNA in artificial lipid membranes (liposomes) can facilitate its uptake and cellular transport. The primary advantages of lipid-based gene transfer methods are ease of preparation and flexibility in substituting different transgene constructs in comparison with the relatively complex process of producing recombinant viral vectors. Cationic liposomes have been used extensively during the last 5 years for cellular delivery of plasmid DNA and antisense oligonucleotides (14). A wide variety of cationic lipid preparations are currently available for DNA transfer both in vitro and in vivo. In addition to cationic lipids, other substances, such as lipopolyamines and cationic polypeptides are now being investigated as potential vehicles for enhanced DNA delivery both for gene transfer and gene blockade strategies (15).

5. Fusigenic-liposome Mediated Gene Transfer (HVJ Liposomes)

This method utilizes a combination of fusigenic proteins of the Sendai virus [Hemagglutinating Virus of Japan (HVJ)] in conjunction with neutral liposomes. HVJ is an RNA virus and belongs to the paramyxovirus family, which has HN and F glycoproteins on its envelope (16). HN binds with glycol-type sialic acid groups that act as receptors on the cell surface, and F protein can interact directly with a cellular lipid bilayer and induce fusion. HVJ liposomes consist of neutral liposomes complexed with UV-light-inactivated HVJ virus. Fusion of HVJ liposome complexes with the cell membrane may result in the release of DNA directly into the cytosol and facilitating nuclear uptake. HVJ-liposome methods have been successfully employed for gene transfer in vivo to many tissues including liver, kidney, and the vascular wall. A major limitation to current HVJ-liposome techniques is the need to undertake a multistep liposome preparation procedure immediately prior to use and the poor long-term stability of the complexes.

6. Other In Vivo Gene Transfer Methods

Plasmids are circular chains of DNA that were originally discovered as a natural means of gene transfer between bacteria. Naked plasmids can also be used to transfer DNA into mammalian cells. The direct injection of plasmid DNA into tissues in vivo can result in transgene expression. Plasmid uptake and expression, however, has generally been achieved at reasonable levels only in skeletal and myocardial muscle (17). The uptake of naked oligonucleotides is also inefficient after either intravascular administration or direct injection. Various catheters that have been designed to enhance local drug delivery to isolated segments of target vessels have been proposed as vehicles for local vascular gene therapy. The controlled application of a pressurized environment to vascular tissue in a nondistended manner has recently been found to enhance oligonucleotide uptake and nuclear localization. This method may be particularly useful for ex vivo applications such as vein grafting or transplantation, and may represent a means of enhancing plasmid gene delivery, as well (18).

7. Regulating Transgene Expression

In addition to effective gene delivery, many therapeutic settings will demand some degree of control over the duration, location, and degree of transgene expression. To this end, researchers have developed early gene promoter systems that allow the clinician to regulate the spatial or temporal pattern of gene expression. These systems include tissue-specific promoters that have been isolated from genetic sequences encoding proteins with natural restriction to the target tissue, such as the von Willebrand factor promoter in endothelial cells and the a-myosin heavy chain promoter in myocardium (19). Promoters have also been isolated from nonmammalian systems that can either promote or inhibit downstream gene expression in the presence of a pharmacological agent such as tetracycline, zinc, or steroids (20). In addition, regulation of transgene expression may even be relegated to the physiological conditions, with the incorporation of promoters or enhancers that respond to specific conditions such as hypoxia or increased oxidative stress (21).

II. GENE THERAPY FOR HYPERLIPIDEMIA AND SYSTEMIC DISEASES

Hyperlipidemia, in both its acquired and inherited forms, has been clearly shown to be an independent risk factor for the development of atherosclerotic vascular disease, myocardial infarction, and stroke. The use of drug therapies in the treatment of acquired forms of this disease, through either 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitors or bile acid binding resins, has proven effective in decreasing the morbidity and mortality associated with hyperlipidemia. However, these same regimens have had their limitations in the treatment of inherited disorders such as familial hypercholesterolemia (FH) and apo E deficiency. The shortcomings of conventional pharmacological therapy have therefore stimulated an interest in the genetic replacement of these missing proteins in hepatocytes as a potential means of reducing cholesterol levels in patients with these rare disorders.

A. Ex Vivo Approach

Familial hypercholesterolemia, caused by mutations in the low density lipoprotein (LDL) receptor, is the most common ge-

netically linked source of hypercholesterolemia, and the heterozygous form is the most common inherited cardiovascular disease (22). The receptor functions to facilitate the uptake of LDL in the liver and is essential for maintaining normal cholesterol levels. In a rabbit model of LDL receptor deficiency, Chowdhury et al. surgically harvested autologous hepatocytes, and stably transferred the gene for the LDL receptor into those cells in culture using a replication-deficient retrovirus. The cells were then infused back into the animals' liver via the portal circulation, and ingraftment of the genetically engineered cells into the liver succeeded in lowering plasma cholesterol levels (23). These encouraging results spawned several clinical trials, the results of which were not as dramatic (24,25). Despite a 30% reduction in serum LDL levels for up to 6 months in the rabbit model, therapeutic reductions in LDL in the clinical trials ranged from 0–15%, with a significantly shorter duration. These differences likely reflect the need for a much higher number of genetically modified autologous cells in the human subject to produce significant therapeutic changes. The immunological response to these infected cells cultured in media containing nonhuman proteins may have also played a role in reducing the duration of clinical efficacy.

B. In Vivo Approach

Researchers have shown that after intravenous injection of adenovirus, more than 90% of transgene expression occurs in the liver. This finding led to the investigation of an in vivo approach to the genetic treatment of this FH. Several groups have reported complete correction of elevated LDL levels in mouse and rabbit models of FH after injection of a replication-deficient adenovirus containing the LDL receptor gene (26–29). However, these effects were found to be transient, once again likely due to the immune responses directed both to adenoviral proteins as well as to the transgene product. Additionally, Kozarsky et al. has reported effective adenoviral-mediated gene transfer of the VLDL receptor in a mouse model for FH with transient correction of hypercholesterolemia (30). Apo E-deficiency has been shown to lead to marked hypercholesterolemia, hypertriglyceridemia, and increased levels of cholesterol-rich VLDL and chylomicron remnants. Apo E-deficient mice treated by adenovirus-mediated gene transfer of the human apo E3 gene resulted in a shift in the plasma lipoprotein distribution from primarily VLDL and LDL in the control mice to predominantly HDL in transfected mice (31). Furthermore, in normal mice, adenovirus-mediated transfer of a gene encoding apo A-I produced transient, physiologically relevant elevations of HDL cholesterol that were comparable to elevations observed in transgenic animals that overexpressed the apo A-I gene (32).

The progress achieved in the treatment of experimental FH through the in vivo genetic manipulation of hepatocytes has brought researchers a step closer toward the permanent correction of this devastating disease. With the development and/or improvement of gene delivery vector systems that can produce long-term, stable integration and that do not lead to a significant inflammatory or immune response, one can also foresee a time where other systemic diseases like diabetes and coagulopathies such as hemophilia can be cured.

III. GENE THERAPY FOR VASCULAR DISEASES

A. Gene and Oligonucleotide Therapy of Restenosis

Recurrent narrowing of arteries following percutaneous angioplasty, atherectomy, or other disobliterative techniques is a common clinical problem that severely limits the durability of these procedures for patients with atherosclerotic occlusive diseases. In the case of balloon angioplasty, restenosis occurs in approximately 30–40% of treated coronary lesions and 30–50% of superficial femoral artery lesions within the first year. Intravascular stents reduce the restenosis rates in some settings; however, the incidence remains significant and long-term data are limited. Despite impressive technological advances in the development of minimally invasive and endovascular approaches to treat arterial occlusions, the full benefit of these gains awaits the resolution of this fundamental biological problem.

Restenosis is an attractive target for gene therapy not only because of its frequency (and its associated costs incurred on the health care system) but more so because it is a local tissue reaction that develops precisely at a site of intervention to which access has already been accomplished. A potential advantage of the genetic approach over more conventional pharmacotherapies is that a single dose of a gene therapy agent may have a protracted biological effect. It has been reasoned that the appropriate genetic modification, performed locally at the time of angioplasty, could induce a long-term benefit in patency by altering the healing response. The potential role for gene therapy in the prevention of restenosis will depend on the identification of an appropriate molecular target, a suitable vector system for efficiently targeting vessel wall cells, and methods of achieving local delivery without producing undue damage or distal tissue ischemia. Presently, considerable hurdles remain despite significant progress in each of these areas.

The pathophysiology of restenosis reflects a paradigm of the healing response of arteries that are injured by reconstructive techniques. It is comprised of a contraction and fibrosis of the vessel wall known as remodeling, and an active growth of a fibrocellular lesion composed primarily of VSMC and extracellular matrix. The latter process, known as neointimal hyperplasia, involves the stimulation of the normally ''quiescent'' VSMCs in the arterial media into the ''activated'' state characterized by rapid proliferation and migration. A number of growth factors are believed to play a role in the stimulation of VSMCs during neointimal hyperplasia, including platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), and angiotensin II (33). Activated VSMCs have also been found to produce a variety of enzymes, cytokines, adhesion molecules, and other proteins that not only enhance the

inflammatory response within the vessel wall but also stimulate further vascular cell abnormality (34–37).

Although it is now thought that remodeling may account for the majority of late lumen loss after balloon dilation of atherosclerotic vessels, proliferation has been the predominant target of experimental genetic interventions. There have been 2 general approaches: cytostatic, in which cells are prevented from progressing through the cell cycle to mitosis, and cytotoxic, in which cell death is induced. A group of molecules known as cell cycle regulatory proteins act at different points along the cell cycle, mediating progression toward division. It has been hypothesized that by blocking expression of the genes for 1 or more of these proteins, one could prevent the progression of VSMCs through the cell cycle and inhibit neointimal hyperplasia. Morishita et al. demonstrated near-complete inhibition of neointimal hyperplasia after carotid balloon injury via HVJ-liposome-mediated transfection of the vessel wall with a combination of antisense ODN against cell cycle regulatory genes (38,39). Arrest of the cell cycle via antisense blockade of either of 2 proto-oncogenes, c-myb or c-myc, has been found to inhibit neointimal hyperplasia in models of arterial balloon injury (40,41), although the specific antisense mechanism of the ODN used in these studies has subsequently been questioned (42,43).

In addition to transfection of cells with antisense ODN, cell cycle arrest can also be achieved through manipulation of transcription factor activity. The activity of a number of cell cycle regulatory genes is influenced by a single transcription factor known as E2F (44). In quiescent cells, E2F is bound to a complex of other proteins, including a protein known as the retinoblastoma gene product (Rb), that prevents its interaction with chromosomal DNA and its stimulation of gene activity. In proliferating cells, E2F is released, resulting in cell-cycle, gene activation. A transcription factor decoy bearing the consensus-binding sequence recognized by E2F can be employed as a means to inhibit cellular proliferation (Fig. 1). Morishita et al. demonstrated the use of this strategy to prevent VSMC proliferation and neointimal hyperplasia after rat carotid balloon injury (45). Alternatively, Chang et al. showed that localized arterial infection with a replication-defective adenovirus encoding a nonphosphorylatable, constitutively active form of Rb at the time of balloon angioplasty significantly reduced smooth muscle cell proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis (46). Similar results were also obtained by adenovirus-mediated overexpression of a ''natural'' inhibitor of cell cycle progression, the cyclin-dependent kinase inhibitor, p21, that likely prevents hyperphosphorylation of Rb in vivo (47). In addition to the blockade of cell-cycle, gene expression, interruption of mitogenic signal transduction has been achieved in experimental models as well. For example, *Ras* proteins are key transducers of mitogenic signals from membrane to nucleus in many cell types. The local delivery of DNA vectors expressing *Ras* dominant negative mutants, which interfere with *Ras* function, reduced neointimal lesion formation in a rat carotid artery balloon injury model (48).

Nitric oxide mediates a number of biological processes that are thought to mitigate neointima formation in the vessel wall, such as inhibition of VSMC proliferation, reduction of platelet adherence, vasorelaxation, promotion of endothelial cell survival, and possible reduction of oxidative stress. In vivo transfer of plasmid DNA coding for endothelial cell nitric oxide synthase (ecNOS) has been investigated as a potential paracrine strategy to block neointimal disease. EcNOS cDNA driven by a β-actin promoter and CMV enhancer was transfected into the VSMCs of rat carotid arteries after balloon injury. This model is known to have no significant regrowth of endothelial cells within 2–3 weeks after injury and therefore capable of loss of endogenous ecNOS expression. Results revealed expression of the transgene in the vessel wall, along with improved vasomotor reactivity and a 70% inhibition of neointima formation (Fig. 2) (49).

An example of a direct cytotoxic approach to the prevention of neointima formation is the transfer of a suicide gene such as the herpes simplex virus-TK (HSV-TK) gene into VSMCs. Using an adenoviral vector, HSV-TK was introduced into the VSMCs of porcine arteries rendering the smooth muscle cells sensitive to the nucleoside analog ganciclovir given immediately after balloon injury. After 1 course of ganciclovir treatment neointimal hyperplasia decreased by about 50% (7). More recently, Pollman and associates (50) induced endogenous machinery for VSMC ''suicide'' in a strategy designed to inhibit the growth or achieve regression of neointimal lesions. This strategy involved antisense ODN blockade of a ''survival'' gene known as Bcl-x, which helps protect cells from activation of programmed cell death, or apoptosis.

Another potentially relevant biological target for treatment of restenosis is reendothelialization, which might be accelerated by local delivery of a proangiogenic factor [e.g., vascular endothelial cell growth factor (VEGF)] at the angioplasty site. This is the basis for the only current U.S. clinical trial of gene therapy for the prevention of restenosis in the peripheral circulation, in which the human VEGF gene is administered as a ''naked'' circular DNA plasmid directly to the injured arterial wall on the surface of the angioplasty balloon (51). The investigators hypothesize that the low efficiency of this delivery method is balanced by the high biological potency of this secreted, angiogenic cytokine, enabling a significant local biological effect despite poor gene transfer.

Successful and efficient gene transfer to the injured, atherosclerotic, arterial wall presents unique mechanical and kinetic challenges. For strategies designed to attenuate or prevent VSMC proliferation, the target cell mass lies within the media of the vessel wall. Following balloon angioplasty, mechanical disruption and dissection of plaque may facilitate particle delivery to deeper layers of the vessel wall; however, uniform gene delivery to the bulk of target VSMCs has been difficult to achieve despite the development of a number of specialized local delivery catheters. Experimental models of neointima formation in animals may not be clinically relevant given their high, uniform, cellular content and absence of the more pre-

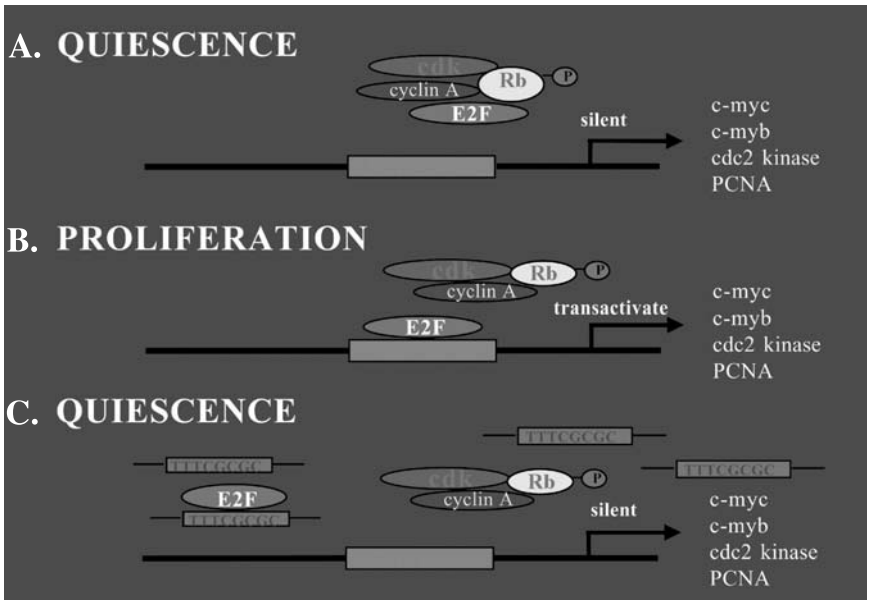

Figure 1 Principle of E2F ''decoy'' strategy. TTTCGCGC, consensus sequence for the E2F binding site. (A) In quiescent cell state, the transcription factor E2F is complexed Rb (retinoblastoma gene product), cyclin A, and cyclin-dependent kinase cdK2. (B) Phosphorylation of releases free E2F, which binds to cis elements of the cell-cycle regulatory genes, resulting in the transactivation of these genes. (C) The E2F decoy cis-element double-stranded oligonucleotide binds to free E2F, preventing E2F-mediated transactivation of cell-cycle regulatory genes. See the color insert for a color version of this figure.

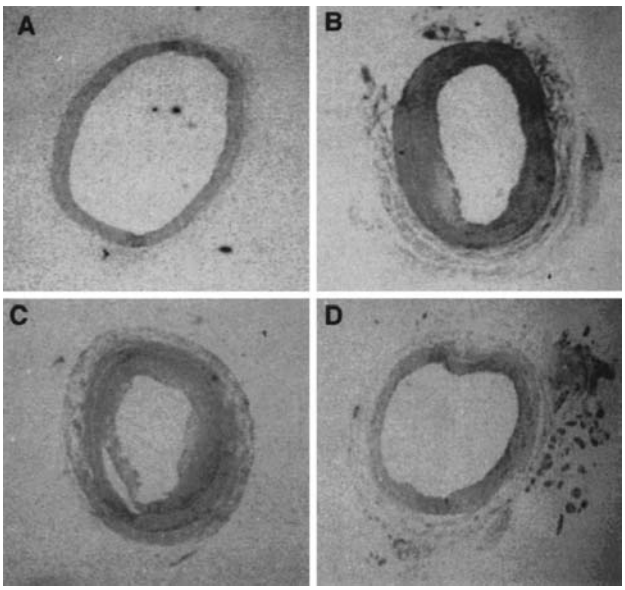

Figure 2 Inhibition of neointimal hyperplasia by in vivo gene transfer of endothelial cell-nitric oxide synthase (ecNOS) in balloon-injured rat carotid arteries. (A) uninjured control artery (CTRL); (B) injured, untransfected artery (INJ); (C) injured, control vector transfected artery (INJ + CV); (D) injured, ecNOS transfected artery (INJ + NOS).

dominant noncellular components of complex atherosclerotic plaque in humans.

An ex vivo gene transfer approach, involving implantation of genetically modified endothelial cells or VSMCs at sites of arterial injury, is also being investigated. Conte et al. successfully demonstrated efficient repopulation of denuded rabbit arteries with genetically modified autologous endothelial cells (52). The need to harvest autologous donor tissue for target cells, coupled with the increased costs and complexity of tissue culture, have greatly dampened the enthusiasm for this strategy in favor of more direct methods. Nonetheless, it remains clinically feasible and, in the case of endothelial cells, the implanted cells alone may confer beneficial properties to the healing arterial wall. Application of these cell transplantation approaches would be greatly facilitated by the development of ''universal donor'' cell lines in which major histocompatibility antigens have been ''knocked out.'' Such a development, while clearly years or decades away, may no longer be merely science fiction fantasy.

In summary, it would appear that the application of gene therapy for post angioplasty restenosis may be somewhat premature (53). In addition to major obstacles in delivering gene transfer agents to the atherosclerotic vessel wall, the fundamental biological process remains incompletely understood. Nonetheless, continued progress on each of these fronts warrants an optimistic view for genetic approaches to control the arterial injury response. These developments will undoubtedly yield important corollaries for the surgical treatment of arterial occlusive diseases as well.

B. Gene Therapy vs. Molecular Therapy for Angiogenesis

The vascularization observed in neoplastic tissue led researchers such as Judah Folkman in the 1970s to investigate the role of molecular factors in the induction of new blood vessel growth (54). The subsequent identification and characterization of ''angiogenic'' growth factors created an opportunity not only to target the growth of solid tumors, but also to attempt the therapeutic ''neovascularization'' of tissue rendered ischemic by occlusive disease in the native arterial bed. Angiogenesis has come to refer more strictly to the sprouting of new capillary networks from preexisting vascular structures, whereas vasculogenesis is the de novo development of both simple and complex vessels during embryonic development. Although it has been clearly established in a number of animal models that angiogenic factors can, in fact, stimulate the growth of capillary networks in vivo, it is less certain that these molecules can induce the development of larger, more complex vessels in adult tissues that would be capable of carrying significantly increased bulk blood flow. Nevertheless, the possibility of an improvement even of just the microvascular collateralization as a ''biological'' approach to the treatment of tissue ischemia has sparked the beginning of human clinical trials in neovascularization therapy.

After the first description of the angiogenic effect of fibroblast growth factors (FGFs), an abundance of ''proangiogenic'' factors were discovered to stimulate either endothelial cell proliferation, enhanced endothelial cell migration, or both. Many of these factors possess heparin-binding domains, which not only increase their retention in heparin-rich extracellular matrix, but also play critical roles in mediating the interaction of the factors with cell surface receptors. Although the list of angiogenic factors includes such diverse molecules as insulin-like growth factor, hepatocyte growth factor, angiopoeitin and platelet-derived endothelial growth factor, the molecules that have received the most attention as potential therapeutic agents for neovascularization are vascular endothelial growth factor (VEGF) and two members of the FGF family, acidic FGF (FGF-1) and basic FGF (FGF-2).

Whereas all angiogenic factors share some ability to stimulate capillary growth in classical models such as the chick allantoic membrane, much debate persists regarding the optimum agent and the optimum route of delivery for angiogenic therapy in the ischemic human myocardium or lower extremity. VEGF may be the most selective agent for stimulating endothelial cell proliferation, although VEGF receptors are also expressed on a number of inflammatory cells, including members of the monocyte-macrophage lineage (55). This selectivity has been viewed as an advantage, since the unwanted stimulation of fibroblasts and VSMCs in native arteries might exacerbate the growth of neointimal or atherosclerotic lesions. Despite this theoretical selectivity, however, the experimental use of VEGF in animal models has been associated not only with capillary growth, but also the development of more complex vessels involving these other cell types (56). The FGFs are believed to be even more potent stimulators of endothelial cell proliferation, but, as their name implies, are much less selective in their proproliferative action (57).

Optimizing the route of drug delivery depends heavily on the pharmacokinetic properties of the agent. Angiogenesis, however, is a very complex biological process involving multiple cell types engaged in multiple activities, including extracellular tissue dissolution and remodeling, cell proliferation, cell migration, cell recruitment, and programmed cell death. The role of any single agent must be understood within the complicated orchestration of multiple signaling agents and effectors. Despite the large amount of data that has become available in the past two decades, details of the cellular and molecular mechanisms of angiogenesis remain poorly understood. Still, it is believed that many of the known angiogenic factors, including VEGF and the FGFs, are exquisitely potent and would not, therefore, require large or prolonged dosing regimens. These conclusions are partly based on the results of in vivo experiments in which a broad range of dosing strategies, ranging from implantation of sustained release formulations to single intra-arterial boluses, have been reported to induce similarly successful increases in tissue perfusion (55).

The contribution of gene therapy to the potential development of therapeutic neovascularization is primarily one of drug delivery. The availability of the genetic sequences encoding these paracrine peptide agents provides an opportunity for the establishment of local tissue factories for drug production. Both intravascular as well as extravascular modes of gene product delivery are feasible, as gene transfer can be attempted either in the walls of vessels feeding the ischemic tissue or in the target myocardial or skeletal tissue itself. In fact, muscle tissue of both myocardial and skeletal origin are among the most receptive for gene transfer with the simplest of agents, pure plasmid DNA (58). Adenoviral vectors are also effective at achieving transgene expression in these muscle cells. A number of reports have suggested that plasmid injections can result in long-term gene expression in these muscle tissues, whereas the higher levels of expression associated with adenoviral vectors is likely limited to 1 or 2 weeks (18,59).

Preclinical studies of angiogenic gene therapy have utilized a number of models of chronic ischemia. An increase in capillary density was reported in an ischemic rabbit hind limb model after VEGF administration, and these results did not differ significantly regardless of whether VEGF was delivered as a single intra-arterial bolus of protein, plasmid DNA applied to surface of an upstream arterial wall, or direct injection of the plasmid into the ischemic limb (55). Direct injection of an adenoviral vector encoding VEGF also succeeded in improving regional myocardial perfusion and ventricular fractional wall thickening at stress in a model of chronic myocardial ischemia induced via placement of a slowly occluding Ameroid constrictor around the the circumflex coronary artery in pigs (60).

Unlike VEGF, FGF-1 and -2 do not possess signal sequences that facilitate secretion of the protein, so that transfer of these genetic sequences is less likely to yield an adequate supply of growth factor to target endothelial cells. To overcome this limitation, Tabata and associates constructed a

plasmid encoding a modified FGF-1 molecule onto which a hydrophobic leader sequence had been added to enhance secretion (61). Delivery of this plasmid to the femoral artery wall, even at very low transfection efficiencies, was found to improve capillary density and reduce vascular resistance in the ischemic rabbit hind limb. Applying a similar strategy, Giordano et al. employed intracoronary infusion of 10^{11} viral particles of an adenoviral vector encoding human FGF-5, which does contain a secretory signal sequence at its amino terminus, to achieve enhanced wall thickening with stress and a higher number of capillary structures per myocardial muscle fiber 2 weeks after gene transfer (62).

Another novel approach to molecular neovascularization has been the combination of growth factor gene transfer with a potentially synergistic method of angiogenic stimulation: transmyocardial laser therapy. The formation of transmural laser channels, though not yet fully established as an effective means of generating increased collateral flow, has had documented, clinical success in reducing angina scores and improving myocardial perfusion in otherwise untreatable patients. In a porcine Ameroid model, Sayeed-Shah et al. found that direct injection of plasmid DNA encoding VEGF in the region surrounding laser channel formation yielded better normalization of myocardial function than either therapy alone (63), and this therapeutic strategy can now be delivered either through minimally invasive thoracotomy or a percutaneous catheter-based approach (Fig. 3).

A number of Phase I safety studies have already been reported in which angiogenic factors or the genes encoding these factors have been administered to patients in small numbers (64,65). These studies have involved either the use of angiogenic factors in patients with peripheral vascular or coronary artery disease who were not candidates for conventional revascularization therapies, or the application of proangiogenic factors as an adjunct to conventional revascularization. The modest doses of either protein factors or genetic material delivered in these studies were not associated with any acute toxicities. Concerns remain, however, regarding the safety of potential systemic exposure to molecules known to enhance the growth of possible occult neoplasms, or that can enhance diabetic retinopathy and potentially even occlusive arterial disease itself. Despite early enthusiasm, there is also little experience with the administration of live viral vectors in extremely large numbers to a large number of patients, and it is uncertain whether potential biological hazards of reversion to replication-competent states or mutation and recombination will eventually become manifest.

In addition to issues of safety, it is also unclear whether the clinical success of conventional revascularization, which has involved the resumption of lost bulk blood flow through larger conduits, will be reproduced via biological strategies that primarily involve increased microscopic collateral networks. It must also be remembered that neovascularization is itself a naturally occurring process, and that the addition of a single factor may not overcome conditions that have resulted in an inadequate endogenous neovascularization response in patients suffering from myocardial and lower limb ischemia. Despite these limitations, angiogenic gene therapy may provide an alternative not currently available to a significant number of patients suffering from untreatable disease, and may offer an adjunct to traditional therapies that improves their long-term outcomes.

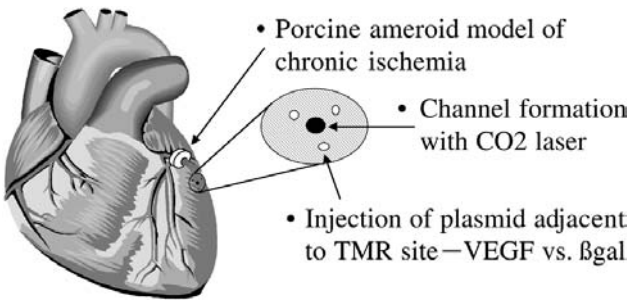

Figure 3 Combined gene transfer and transmyocardial laser revascularization (TMR). Schematic representation of chronic ischemia induced by placement of Ameroid constrictor around the circumflex coronary artery in pigs. Ischemic hearts that underwent TMR, followed by injection of plasmid encoding VEGF, demonstrated better normalization of myocardial function than either therapy alone. See the color insert for a color version of this figure.

IV. GENE THERAPY FOR VASCULAR GRAFTS

A. Vein Graft Engineering with E2F Decoy ODN

The long-term success of surgical revascularization in the lower extremity and coronary circulations has been limited by significant rates of autologous vein graft failure. No pharmacological approach has been successful at preventing long-term graft diseases such as neointimal hyperplasia or graft atherosclerosis. Gene therapy offers a new avenue for the modification of vein graft biology that might lead to a reduction in clinical morbidity from graft failures. Intra-operative transfection of the vein graft also offers an opportunity to combine intact tissue DNA transfer techniques with the increased safety of ex vivo transfection, and a number of studies have documented the feasibility of ex vivo gene transfer into vein grafts using viral vectors.

The vast majority of vein graft failures have been linked to the neointimal disease is part of graft remodeling after surgery (66). Although neointimal hyperplasia contributes to the reduction of wall stress in vein grafts after bypass, this process can also lead to luminal narrowing of the graft conduit during the first years after operation (67). Furthermore, the abnormal neointimal layer, with its production of proinflammatory proteins, is believed to form the basis for an accelerated form of atherosclerosis that causes late graft failure (68).

Similar to observations made in the arterial balloon injury model, it was found that a combination of antisense ODN that inhibit expression of at least two cell cycle regulatory genes could significantly block neointimal hyperplasia in vein grafts (69). Additionally, E2F decoy ODN yielded similar efficacy in the vein graft when compared to the arterial injury model (70). In contrast to arterial balloon injury, however, vein grafts are not only subjected to a single injury at the time of operation, but are also exposed to chronic hemodynamic stimuli for remodeling. Despite these chronic stimuli, a single, intraoperative ODN treatment of vein grafts resulted in a resistance to neointimal hyperplasia that lasted for at least 6 months in the rabbit model (69). During that time period, the grafts treated with antisense ODN were able to adapt to arterial conditions via hypertrophy of the medial layer. Furthermore, these genetically engineered conduits proved resistant to diet-induced graft atherosclerosis (Fig. 4) and were associated with preserved endothelial function (71).

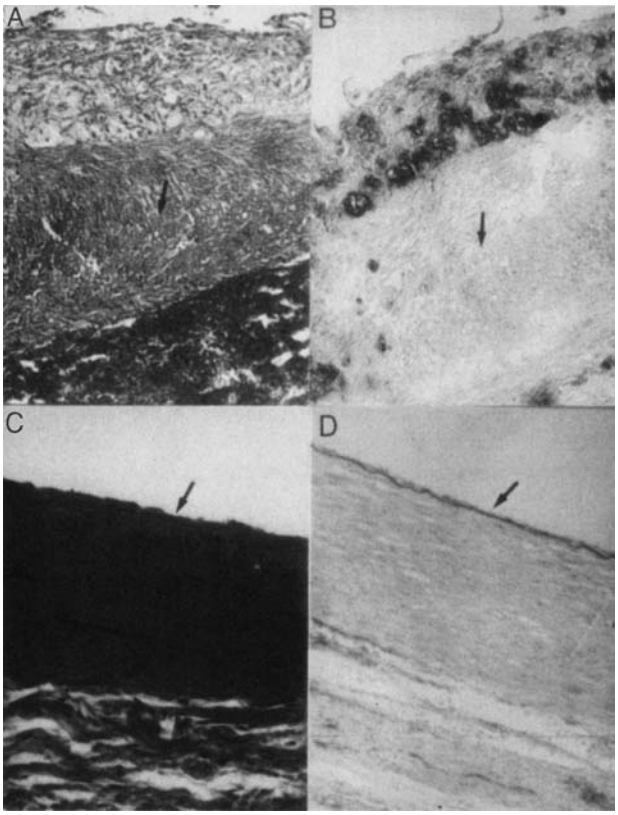

Figure 4 Control oligonucleotide-treated (A and B) and antisense oligonucleotide (against cdc2 kinase/PCNA)-treated vein grafts (C and D) in hypercholesterolemic rabbits, 6 weeks after surgery ($\times$ 70). Sections were stained with hematoxylin/van Gieson (A and C) and a monoclonal antibody against rabbit macrophages (B and D). Arrows indicate the location of the internal elastic lamina.

A large-scale, prospective, randomized, double-blind trial of human vein graft treatment with E2F decoy ODN has been initiated (18). Efficient delivery of the ODN is accomplished within 15 min during the operation by placement of the graft after harvest in a device that exposes the vessel to ODN in physiological solution and creates a nondistending, pressurized environment of 300 mm Hg. Preclinical findings indicated ODN delivery to greater than 80% of graft cells, and effective blockade of target gene expression. This study will measure the effect of cell-cycle gene blockade on primary graft failure rates, and represents 1 of the first attempts to definitively determine the feasibility of clinical genetic manipulation in the treatment of a common cardiovascular disorder.

B. Vein Graft Gene Transfer

With the development of viral-mediated gene delivery methods, some investigators have begun to explore the possibility of using these systems ex vivo in autologous vein grafts. Chen et al. (72) demonstrated the expression of the marker gene b-galactosidase along the luminal surface and in the adventitia of 3-day porcine vein grafts infected with a replication-deficient adenoviral vector at the time of surgery. The vein segments were incubated in a high viral titer suspension for approximately 2 h prior to implantation. Although these researchers documented expression of a soluble vascular cell adhesion molecule-1 (sVCAM-1) isomer on the luminal and abluminal surfaces of 3-day grafts infected with an adenoviral vector encoding this protein, no long-term expression or functional effect of this gene was reported. In a study previously alluded to, Kupfer et al. (73) explored the use of a novel adenovirus-based transduction system, in which adenoviral particles were linked to plasmid DNA via biotin/streptavidin-transferrin/polylysine complexes. β-galactosidase expression was documented 3 and 7 days after surgery in rabbit vein grafts that had been incubated for 1 h with complexes prior to grafting. Expression was again greatest on the luminal surfaces of the grafts, although then presence of transfected cells in the medial and adventitial layers was also reported.

The feasibility of gene transfer in vein grafts has subsequently led to the investigation of potential therapeutic endpoints such as neointima formation. George et al. (74), using a replication-deficient adenovirus expressing tissue inhibitor of mettaloproteinasee-2 (TIMP-2), was able to demonstrate a dramatic decrease in neointimal formation in a saphenous vein organ culture model. In vivo gene transfer has also been shown to effectively reduce neointima formation in experimental vein grafts. Bai et al. (75) performed intra-operative transfection of the senescent cell-derived inhibitor (sdi-1) gene, a downstream mediator of the tumor suppresser gene p53, using the HVJ-liposome system, and was once again able to demonstrate a significant reduction in neointima formation. The use of gene transfer in vein grafts may reach beyond the treatment of the graft itself. The expression of therapeutic proteins by transduced grafts can lead to the treatment of diseases in tissues downstream to the location of graft implantation, further expanding the versatility of this bypass conduit.

C. Bioprosthetic Grafts

Prosthetic materials, such as PTFE or Dacron, often used as small-caliber arterial substitutes or in the construction of arteriovenous grafts, have been limited in their long-term use due to their thrombogenic surfaces. A bioengineering, cell-based strategy for decreasing or eliminating this thrombogenicity may therefore yield a prosthetic graft capable of maintaining normal flow. Successful isolation of autologous endothelial cells and their seeding onto prosthetic grafts in animal models has been well characterized (76). Furthermore, it has been hypothesized that one can enhance the function of these endothelial cells via the transfer of genes prior to seeding of the cells on the graft surface. Such a bioprosthesis could be useful for delivering genetically engineered factors that would enhance graft function and survival or even provide an avenue for intravascular drug delivery. First indication for the possible use of this strategy was presented by Wilson at al. (10), who demonstrated successful endothelialization of a prosthetic vascular graft with autologous endothelial cells transduced with a recombinant retrovirus encoding the *lacZ* gene. Additionally, seeding of transduced vascular smooth muscle cells into the interstices of a PTFE graft then luminally seeded with untreated endothelial cells revealed stable expression of the reporter gene after 3–5 weeks (77).

Successful clinical applications of these concepts, however, have not been reported. In an attempt to decrease graft thrombogenicity, Dunn et al. (78) seeded 4-mm Dacron grafts with retrovirally transduced endothelial cells encoding the gene for human tissue plasminogen activator (TPA) and implanted them into the femoral and carotid circulation of sheep. The proteolytic action of TPA resulted in a decrease in seeded endothelial cell adherence, with no improvement in surface thrombogenicity. The use of VEGF in this context also has potentially significant clinical applications. VEGF, a potent endothelial cell mitogen, when transduced into a limited number of endothelial cells and placed on the graft surface, may promote endothelial survival and replication, and yield improved and more rapid graft coverage with a nonthrombogenic endothelial layer. Additionally, secretion of VEGF could lead to angiogenesis distal to the grafted area in what is likely to be an ischemic tissue bed. Yamamoto et al. (79) has demonstrated successful seeding of PTFE grafts with VEGF-transduced, adipose-derived, endothelial cells and expression of the transgene after several weeks. Further studies are needed to determine the local effect of VEGF secretion on endothelial cell proliferation along with distant angiogenic stimuli.

V. GENE THERAPY FOR THE HEART

Failure of the myocardium due to insults such as ischemia, infection, metabolic disorders, or substance abuse afflicts millions of Americans annually. Traditional pharmacotherapy and surgical intervention has succeeded in ameliorating these problems, but the advent of gene transfer technology has brought a heightened interest in either correcting, preventing, or limiting the functional deficits sustained by the myocardium. The myocardium has been shown to be receptive to the introduction of foreign genes. As seen in noncardiac muscle (80), measurable levels of gene activity have been found after direct injection of plasmids into myocardial tissue in vivo (81). Although limited to a few millimeters surrounding the injection site, these observations have laid the basis for consideration of gene transfer as a therapeutic approach to cardiac disease. The distribution of myocardial expression of genes after direct injection in rats has been enhanced via incorporation of the gene into an adenoviral vector (59). Additionally, both adenoviral and adeno-associated viral vectors can be delivered to the myocardial and coronary vascular cells via intracoronary infusion of highly concentrated preparations in rabbits and porcine models, respectively (82,83). Gene transfer into the myocardium has also been achieved via either the direct injection or intracoronary infusion of myoblast cells that have been genetically engineered in cell culture (84).

A. Gene Therapy to Enhance Contractility

The β-adrenergic receptor (β-AR) is known to be a critical player in mediating the ionotropic state of the heart and has received significant attention as a target for genetic therapeutic intervention in congestive heart failure. Milano et al. (85), using transgenic mice expressing the β_2-AR under the control of the cardiac α-MHC promoter, demonstrated an approximately 200-fold increase in the level of β_2-AR along with highly enhanced contractility and increased heart rates in the absence of exogenous β-agonists. This genetic manipulation of the myocardium has generated considerable interest toward the use of gene transfer of the β-AR gene into the ailing myocardium as a means of therapeutic intervention. To date, attempts at exploring this exciting possibility have been primarily limited to cell culture systems. Akhter et al. (86) successfully demonstrated improved contractility in rabbit ventricular myocytes that have been chronically paced to produce hemodynamic failure after adenoviral-mediated gene transfer of the human β_2-AR. An enhanced chronotropic effect resulting from the injection of a β_2-AR plasmid construct into the right atrium of mice has been demonstrated by Edelberg et al. (87), but no evaluation of enhanced contractility by transfer of this gene into the ventricle has been reported. These results demonstrate the feasibility of using the β-adrenergic pathway and its regulators as a means by which to treat the endpoint effect of the variety of cardiac insults that exist.

There has also been recent interest in the enhancement of contractility through the manipulation of intracellular calcium levels. Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) transporting enzyme, which regulates Ca^{2+} sequestration into the sarcoplasmic reticulum (SR), has been shown to be decreased in a variety of human and experimental cardiomyopathies. Using adenoviral-mediated gene transfer, Hajjar et al. (88) was able to overexpress the SERCA2a protein in neonatal rat cardiomyocytes. This led to an increase in the peak $[Ca^{2+}]_i$ release, a decrease in resting $[Ca^{2+}]_i$ levels, and more importantly to enhanced contraction of the myocardial cells as detected by shortening measurements. The success of this ap-

proach to improving myocardial contractility has yet to be documented in vivo, but once again, provides a novel and potentially exciting means by which to treat the failed heart.

B. Gene Therapy for Myocardial Infarction

Coronary artery atherosclerosis and resulting myocardial ischemia is a leading cause of death in developed countries. Reperfusion injury has been linked to significant cellular damage and progression of the ischemic insult. In addition to stimulating therapeutic neovascularization, genetic manipulation may be used as a means to limit the degree of injury sustained by the myocardium after ischemia and reperfusion.

The process of tissue damage resulting from ischemia and reperfusion has been well characterized. Briefly, the period of ischemia leads to an accumulation of adenosine monophosphate, which then leads to increased levels of hypoxanthine within and around cells in the affected area. Additionally, increased conversion of xanthine dehydrogenase into xanthine oxidase takes place, which upon exposure to oxygen during the period of reperfusion, converts hypoxanthine to xanthine, leaving behind the cytotoxic oxygen radical, superoxide anion (O_2^-). O_2^- can then go on to form hydrogen peroxide (H_2O_2), another oxygen radical species. Ferrous iron (Fe^{2+}), which accumulates during ischemia, reacts with H_2O_2, which leads to the formation of the most potent oxygen radical, hydroxyl anion (OH^-) (89). These oxygen radicals result in cellular injury via lipid peroxidation of the plasma membrane, oxidation of sulfhydryl groups of intracellular and membrane proteins, nucleic acid injury, and breakdown of components of the extracellular matrix such as collagen and hyaluronic acid (90). Natural oxygen radical scavengers, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and hemoxygenase (HO), function through various mechanisms to remove oxygen radicals produced in normal and injured tissues.

The degree of oxygen radical formation produced after ischemia-reperfusion in the heart can overwhelm the natural scavenger systems. Overexpression of either extracellular SOD (ecSOD) or manganese SOD (MnSOD) in transgenic mice has revealed improved postischemic cardiac function and decreased cardiomyocyte mitochondrial injury in adriamycin-treated mice, respectively (91,92). These findings suggest a role for gene transfer of these natural scavengers as a means by which to protect the myocardium in the event of an ischemia-reperfusion event. Li et al. (93) demonstrated substantial protection against myocardial stunning using intra-arterial injection of an adenovirus containing the gene for Cu/ZnSOD (the cytoplasmic isoform) into rabbits, although no studies have investigated the direct antioxidant effect and ensuing improvement in myocardial function of this treatment after ischemia and reperfusion. This application of gene therapy technology may offer a novel and exciting approach for prophylaxis against myocardial ischemic injury if incorporated into a system of long-term, regulatable transgene expression. In addition to the overexpression of antioxidant genes, some researchers have proposed intervening in the program of gene expression within the myocardium that leads to the downstream deleterious effects of ischemia reperfusion. For example, the transfection of rat myocardium with decoy oligonucleotides that block activity of the oxidation-sensitive transcription factor NFκ-B, linked to expression of a number of proinflammatory genes, succeeded in reducing infarct size after coronary artery ligation (94).

At the cellular level, myocardial infarction results in the formation of a scar that is composed of cardiac fibroblasts. Given the terminal differentiation of cardiomyocytes, loss of cell mass due to infarction does not result in the regeneration of myocytes to repopulate the wound. Researchers have therefore pursued the possibility of genetically converting cardiac fibroblasts into functional cardiomyocytes. The feasibility of this notion gained support from the work of Tam et al. (95), who demonstrated the in vitro conversion of cardiac fibroblasts into cells resembling skeletal myocytes via the forced expression of a skeletal muscle lineage-determining gene, MyoD, using retroviral-mediated gene transfer. Fibroblasts expressing the MyoD gene were observed to develop multinucleated myotubes similar to those seen in striated muscle, which expressed MHC and myocyte-specific enhancer factor-2. Additionally, Murry et al. (96) also showed expression of myogenin and embryonic skeletal MHC after transfection of rat hearts injured by freeze-thaw with an adenovirus containing the *MyoD* gene. At this time, however, functional cardiomyocytes have not yet been identified in regions of myocardial scarring treated with in vivo gene transfer.

C. Gene Therapy for Immunomodulation

Genetic manipulation of donor tissues offers the opportunity to design organ-specific immunosuppression during cardiac transplantation. Although transgenic animals are being explored as potential sources for immunologically protected xenografts (97), the delivery of genes for immunosuppressive proteins, or the blockade of certain genes in human donor grafts, may allow site-specific, localized immunosuppression and a reduction or elimination of the need for toxic systemic immunosuppressive regimens. Gene activity has been documented in transplanted mouse hearts for at least 2 weeks after intra-operative injection of the tissue with either plasmid DNA, or retroviral or adenoviral vectors (98). The transfer of a gene for either TGF-b or interleukin 10 in a small area of the heart via direct injection in this model succeeded in inhibiting cell-mediated immunity and delaying acute rejection (99,100). In another study, the systemic administration of antisense ODN directed against intercellular adhesion molecule-1 (ICAM-1) also prolonged graft survival and induced long-term graft tolerance when combined with a monoclonal antibody against the ligand for ICAM-1, leukocyte function antigen (101,102).

VI. SUMMARY

Gene therapy has begun a gradual ascent from the realm of pure theory, and has entered a period of intense research into

practical clinical applications. Although the first documentation of clinical success remains eagerly sought, gene manipulation strategies appear now to provide a meaningful addition to the tools available in the design of novel approaches to cardiovascular disease. Further refinement of both current gene therapy methodologies as well as the cardiovascular biologist's understanding of the molecular basis of complex disease processes will enhance the likelihood of such success, and may prove essential to its realization. Nevertheless, ''proof of principle'' has been clearly established; a thoughtful and thorough scientific approach has therefore become warranted as these exciting new possibilities continue to expand.

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27

Gene Therapy for Cancer

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I. INTRODUCTION

Gene therapy for cancer has generated great interest for more than a decade and intensive experimental and clinical investigations are underway. In general, it comprises different technologies to deliver a cDNA of choice to cancer cells or to normal tissue for a variety of diagnostic and therapeutic applications. Based on the complex nature of cancer, these technologies are very heterogeneous, as demonstrated by a variety of concepts such as immunomodulation, and the ''suicide strategy'' (i.e., transfer of the cDNA of a prodrug-converting enzyme); gene replacement strategies such as transfer of a tumor suppressor and/or an antioncogene; viral oncolysis; antiangiogenic and antiproteolytic gene therapy; or the delivery of drug-resistant genes into hematopoietic precursor cells. Many experimental approaches are now in clinical evaluation and more than 630 trials have been published or initiated or are pending. More than 60% of these trials are directed against cancer. In the first part of this contribution, I have tried to summarize the current state of the art of the gene therapeutic vectors now available and to review gene therapy principles strictly from the point of view of cancer therapy. In the second part, I have described experimental and clinical approaches for several types of cancer. The peculiarities of the respective cancer types allow or require customized gene therapeutic approaches, particularly in a clinical setting.

This contribution cannot give a complete picture of all current gene therapy approaches and I apologize for any omissions. Cross-references to the most recent literature reviews and to the corresponding chapters in this book are given. Helpful tools for getting an overview of gene therapy trials are found under www.wiley.co.uk/genetherapy/DATABASE at the Genetic Medicine Clinical Trials Database and under www4.od.nih.gov/oba/, a website provided by the NIH, Office of Biotechnology.

II. THERAPEUTIC PRINCIPLES

A. Immunomodulation

It has long been recognized that tumors exhibit to a certain extent immunogenicity (1). As demonstrated by several studies, (for further details on these studies, see Tables 2 and 3 beginning on pg. 551) the human immune system seems to respond to this immunogenicity by recognizing specific tumor antigens and, consequently, by mounting humoral and cellular responses. However, during cancer development, this response is only of limited intensity and duration (2,3). As the mechanisms that cancer cells use to escape detection by the immune system have been elucidated, more and more strategies have been developed to reconstitute an effective antitumor immune response. The tremendous increase in the knowledge of the immunobiology of cancer has made immunological approaches, e.g., immunomodulation, the most dominant strategy in cancer gene therapy during recent years (4). In general, immuno-modulation studies can be categorized according to (a) the target cells (tumor cells, host cells, T-cells, or antigen-presenting cells such as dendritic cells or other cells); (b) the mode of gene delivery (which vector, in vitro, ex vivo, in vivo); or (c) the transferred transgenes (cytokines, costimulatory molecules, tumor-associated antigens). Four examples from the multiplicity of these approaches will be described in more detail.

One of the most attractive target cell types for genetic modification are T-lymphocytes. The application of cytokine-transduced, tumor-infiltrating T-lymphocytes (TILs) (5–8) was among the earliest clinical protocols for gene therapy. More recently, T-lymphocytes have been the target for ex vivo genetic modification by cytokine gene transfer and for redirection by tumor antigen-specific T-cell receptors or chimeric receptor genes (extracellular domain: antigen-binding,

intracellular domain: cell-signaling). They have also been isolated from genetically modified tumors or their draining lymph nodes (9–11). Other approaches enhance T-cell reactivity with antibodies that are targeted directly at the respective receptors on T-cells (12,13). These approaches are complemented by different methods to enhance antigen recognition on the surface of tumor cells.

Another immunological approach to generate a local inflammatory response is the use of short-range communications between immune and nonimmune cells. Cytokine-transfected tumor cells or fibroblasts are transferred, which are then supposed to directly activate specific as well as nonspecific immune cells. In this context, the continuous local release of cytokines has been shown to increase the therapeutic index. Additionally or alternatively, costimulatory molecules can be transferred. In tumor cells where MHC class I or class II molecules or costimulatory molecules such as HLA-B7 are downregulated, the transfer of the corresponding wild-type cDNA can reactivate antigen recognition on the surface of the transduced tumor cells.

During the last decade, more and more tumor-derived antigens have been defined. These became a very interesting target for gene transfer approaches (14). In addition to applications of the corresponding peptides, some of these antigens have been transferred by viral vectors or as naked DNA either directly to the tumors or via dendritic cells, evoking an immune response against the tumor. Detection of new tumor antigens with new powerful methods, such as SEREX (serological analysis of tumor antigens by recombinant expression cloning) (2,15), or methods for detection of differential gene expression such as microarray technologies, holds great promise for the future.

Finally, antibody-based immunotherapy should be mentioned in this context (16), particularly since recombinant antibodies have finally shown marked clinical benefit. The underlying concept of this strategy is to raise monoclonal antibodies against soluble tumor antigens or against antigens that are expressed on the surface of the malignant cells or on the tumor stroma. A cancer gene therapeutic use of antibody genes is probably only a matter of time.

B. Prodrug-Converting Enzymes (''Suicide Strategy'')

The ''suicide strategy'' in oncology combines classical cytotoxic chemotherapy with gene transfer technology. The underlying concept is to limit the action of a known cytotoxic drug to the local area of the tumor lesion. To this end, the cDNA of a prodrug-converting enzyme is delivered into the tumor by a vector system of choice. This is followed by regional or systemic application of the corresponding nontoxic prodrug. Once the prodrug reaches the tumor and is taken up by the tumor cells that express the prodrug-converting enzyme, it is converted into the cytotoxic drug. In conventional chemotherapy, toxic and myeloablative side effects can be dose-limiting. In contrast, when using the suicide gene concept, the cytotoxic effects of the converted drug are mainly restricted to the area of tumor infiltration, and the time of action is limited to the presence of the cancer cells expressing the prodrug-converting enzyme. In addition, the efficacy of the suicide strategy is enhanced by the ''bystander effect.'' This molecular mechanism allows the killing of even uninfected tumor cells in the neighborhood of infected cells due to intercellular communication mediated by gap junctions (for example). However, this mechanism is still not fully understood. There are many different prodrug-converting enzymes under experimental or clinical investigation, and the number of systems developed is increasing rapidly (17). For a more detailed overview of the application of the suicide strategy in cancer gene therapy, please see Chapter 20 by B. O'Malley Jr.

The prodrug-converting enzyme most often used for clinical purposes is the herpes simplex virus thymidine kinase gene (HSV tk). The enzyme thymidine kinase (tk) phosphorylates the prodrug ganciclovir (GCV) to GCV-monophosphate, which is then further phosphorylated to toxic GCV-triphosphate. Inhibition of the DNA polymerase by GCV-triphosphate finally leads to cell death. As demonstrated in various studies, tumor eradication has been achieved even if only 10% of the tumor mass is transduced (18). This effect is probably due to a very potent bystander effect. In preclinical animal experiments, a vector carrying the HSV tk gene is usually applied intratumorally. In several reports using immunocompromised animals, a reduction of tumor volume of more than 50% of the controls was achieved in various experimental settings and even complete remissions were frequently observed. In addition to these experimental studies on immunocompromised animals, reports on immunocompetent animals suggest that the immune system may play a supportive role in the efficacy of this approach (19). Promising results of tumor growth inhibition are not without side effects induced by the HSV tk system. Using an in vivo model of adenovirus-mediated gene transfer to the liver of mice and rats, liver toxicity was observed due to unwanted transduction of normal tissue. This can be eliminated either by targeted vectors or tissue-specific gene expression (20,21).

As in other gene therapy strategies, the capability of the suicide strategy to kill cancer cells in experimental and clinical studies is limited by overall low efficacy of gene transfer in vivo. This is probably the main reason for the overall limited clinical efficacy of the suicide strategy in several Phase I, II, and III clinical trials for several types of cancer. Even with the most efficient vector systems, direct intratumoral vector application only leads to a partial transduction of tumors with a nonhomogenous intratumoral vector distribution. Consequently, low antitumor efficacy is seen, particularly in humans, which present much larger tumor burdens than rodents in a typical laboratory situation. Vector distribution could, however, be improved by changing the anatomical route of vector application (intravesical, intraperitoneal, intrathecal, intraventricular, repeated, and bulk-flow); through the use of conditionally replication-competent vectors; by enhancing the bystander effect through the transfer of connections that

increase the number of gap junctions; and by receptor targeting (see Chapter 3 by D. Curiel).

C. Tumor Suppressor Genes and Anti-Oncogenes

During the last decade, an increasing number of genes has been identified which become dysregulated during carcinogenesis. During a complex and multifactorial process leading ultimately to the macroscopic presence of cancer, genes become dysregulated by different molecular mechanisms including gene deletion, mutation, or promotor silencing. At the end of these processes of genetic alteration, which result in activation or inactivation of multiple genes, the cancer cell proliferates in an uncontrolled fashion. It is not able to go into apoptosis and achieves invasive potential. Genes promoting these processes, like oncogenes, become activated. Genes suppressing the processes, like tumor-suppressor genes, become inactivated.

Current approaches in this area include the inactivation of overexpressed oncogenes by antisense molecules or dominant negative mutants or, alternatively, the reconstitution of cells with tumor-suppressor genes that have been lost or have mutated. The rationale is not so much the reversion of tumor cells back into normal cells. This task would be difficult to accomplish because more than one mutation is usually acquired by the tumor cell during the transformation process. The aim is rather to define the weak point in the cell's regulatory balance and consequently to identify the gene or combination of genes that would have the highest impact on the exertion of cell-cycle arrest or better apoptosis. To this end, it is of crucial importance to acquire a sufficient understanding of the cell's balance with respect to signal transduction, cell cycle regulation, and finally, susceptibility to apoptosis. The targeting of the genes known to be dysfunctional in the respective tumor is usually the most efficient procedure. In some cases, a combination of several genes could increase specificity and efficacy. The main obstacle to this therapeutic approach is the need for a particularly high gene-transfer efficacy. Although a mild bystander effect has been reported in the context of p53 gene transfer (22), a high transfer efficacy is usually necessary for eradication of tumors—at least in those cases where the immune system as an adjuvant is not dramatically activated. Such high transfer efficacies are the exception with current vectors. Therefore, a breakthrough for this highly tumor-specific approach particularly depends on future vector development. Among several tumor suppressor genes that have shown in vivo efficacy, p53 has made it into the clinic and is discussed in more detail (e.g., in the chapter dealing with lung cancer). Besides proapoptotic bax (23) and bcl-x_s (24), caspases (25), PTEN, and BRCA1, those genes involved in the regulation of the G1-phase of the cell cycle, have been evaluated particularly closely in animal experiments. Tumor growth could be inhibited by transfer of wild-type and truncated pRb (26,27), which in its active form binds E2F-1 and prevents entry into S-phase, as well as by the cdk/cyclin inhibitors p16 (28,29) and p21 (30), which mainly keep pRb in its hypophosphorylated, active state. It remains to be shown whether cell-cycle arrest is sufficient for therapy of established tumors or whether a strong induction of apoptosis as sometimes seen by transfer of p16 and p27 (29) is required. Undoubtedly, restoration of tumor cells with p53 or p21 can increase the cells' sensitivity to radiation (31–33) and chemotherapy (34–37). Also, combinations of tumor suppressor genes could prove to have overadditive tumoricidal effects as has been shown for the combination of p53 and p16 (28).

Gene therapy directed against oncogenes or apoptosis suppressors like bcl-2 or bcl-x_l has targeted several types of cancer, and several clinical trials are underway. These are discussed in more detail in Part 4 of this contribution and in Chapter 42 by J.A. Roth. The antioncogene approach is in principle supported by the recent success of small molecular inhibitors of oncogenes: inhibitors of the EGF receptor, the RAS pathway, and the ABL gene (Gleevec). Gene therapy has the potential to compete with small molecules because small molecules still efficiently target only enzymes and receptors, whereas transdominant antioncogenes, antisense oligonucleotides, and (recently) the very promising RNA-interfering nucleotides (38,39) can inhibit virtually any oncogene of relevance.

D. Tumor Lysis by Recombinant Viruses

Since its early beginnings, cancer therapy by viral oncolysis has been one of the most challenging strategies to treat human malignancies (40). The idea that viruses may be used as selective anticancer agents dates back almost a century (41). In 1957, 4 years after their discovery, replicating adenoviruses were used in cancer therapy. Of patients with advanced cervical carcinoma receiving intratumoral or intra-arterial injections of wild-type human adenoviruses, 65% had a marked to moderate local tumor response and only 3 out of 30 patients on steroids had a viral syndrome of short duration (42). The underlying concept of this strategy is to inject the virus directly into the tumor, leading to the transduction of a certain number of cells in which viral replication takes place. Consequently, the infected cells are finally disrupted and viral progeny are released, allowing the spread of the infection and an increase in transfer efficacy. Interest and research in this area of molecular biology has exploded, and the available virus-production techniques and purification techniques have been markedly improved. Thus, much larger amounts of adenovirus can now be applied. However, one of the major concerns has been how to limit the viral replication to the site of the tumor. One strategy to confer tumor-specific replication has been developed that utilizes the dependence of the replicating adenovirus on the genetic status of p53 in the infected host cell. Like many DNA viruses, adenoviruses have also developed specific gene products that seem to counteract apoptosis-inducing molecules such as p53. Among the adenoviral genes, the E1B 55kD gene blocks p53, the best-known inducer of apoptosis and indirect inducer of cell cycle arrest. In an intriguing approach (43), the E1B gene was deleted from the adenoviral genome, allowing viral replication only in p53-negative tumor cells but

creating apoptosis upon viral infection in p53-positive normal cells (43,44). Since more than 50% of common solid tumors lack functional p53, this approach was thought to be widely applicable. In the meantime, both the lack of replication in all p53-positive cells and the potent replication in all p53-negative cells (45,46) have been questioned. It seems that defects further down the p53 pathway, as well as functions of E1B55K independent from inactivation of p53, need to be taken into account. They may perhaps moderately limit the applicability of the approach. Independent of the outcome of this debate, this concept has already stimulated virologists and cancer biologists to develop further generations of conditionally replication-competent adenoviruses. Adenoviral E1A inactivates pRB, the well-known tumor suppressor and inducer of cell cycle arrest, and facilitates adenoviral replication by allowing cellular replication. In an approach similar to the one described above, E1A was mutated so as to no longer bind and inactivate pRb; this should lead to viral replication only in tumor cells that typically have defects in the pRb pathway. This concept has been successfully applied in vitro and in vivo (47–49).

Another way to achieve cancer-specific replication is through the use of tissue-specific or tumor-specific gene expression. The adenoviral E1A region, which controls viral replication through several mechanisms, was placed under the control of a tissue- or cancer-specific promoter instead of the E1A promoter. This approach allowed specific replication in the respective cancer cells and tumors when prostate-specific antigen (PSA) or probasin promoters (50,51); TCF-responsive elements that are preferentially activated in colon cancer; or the MUC1 promoter (which is active in breast carcinoma) (52) were used to drive the E1A gene (53). Besides adenoviruses, other oncolytic viruses have been tested in animal models. Like adenoviruses, Herpes simplex virus (HSV), a neurotrophic DNA virus, directly lyses cells during viral shedding. First-generation HSV contains a 360-bp deletion in the thymidine kinase gene; this seems to prevent replication in quiescent cells but allows replication in rapidly proliferating cells (54,55). Second-generation viruses with additional mutations have been generated to increase tumor-specific replication and reduce neurovirulence (56). Newcastle Disease virus (NDV) is a chicken paramyxovirus associated with minimal disease in humans. Cytotoxicity for numerous human tumor cell lines and resistance of several human fibroblast lines have been reported (57). The mechanism that causes tumor selectivity is not fully resolved, but a failure to mount a protective interferon response may be causally involved. Tumor-cell killing may involve virus replication and direct cell lysis and/or induction of tumor necrosis factor (TNF) secretion as well as increased sensitivity of tumor cells to TNF-mediated killing (58,59). Recently, a clinical trial of intravenous injection of NDV into 67 patients with solid tumors was published. Objective responses occurred at higher doses (60).

Autonomous parvoviruses are DNA viruses with small genomes that depend on helper viruses or specific cellular functions for replication. Their cytopathic effect seems to be dependent on DNA replication as well as on the expression of nonstructural gene products and enhanced sensitivity to their effects (56).

In the future, the tremendous increase in knowledge in the field of tumor and virus biology can be used to create appropriate vectors. Several factors that potentially influence clinical efficacy have already been defined. Among these are (a) characteristics of the viruses, such as the size, the time between infection and lysis, as well as the number of virions produced or the induction of a humoral or cellular immune response; and (b) characteristics of the tumor such as distribution of the vasculature or physical barriers to virus spread such as fibrosis. An example of how to surmount such a natural barrier is the demonstration that inactivation of particular adenoviral serotypes by preformed antibodies can be circumvented if other serotypes are used (61).

E. Antiangiogenic and Antiproteolytic Gene Therapy

These types of gene therapy differ markedly from the majority of the other nonimmunological gene cancer therapeutic approaches because the products of the delivered genes act extracellularly. This fact obviates the need to transduce the majority of tumor cells and even allows one to target normal tissue, which may be easier to transduce than the tumor. In addition, these approaches are usually appropriate for long-term treatments. Therefore, the conventional demands of cancer gene therapy—high efficacy of gene transfer, acceptance of immunogenicity, and sufficiency of short duration of gene expression—are replaced by requirements more typical for the treatment of monogenetic disease. These include low immunogenicity of vector and transgene, long-duration gene expression, and achievement of at least threshold levels of gene product in the target tissue or in the blood.

Inhibition of angiogenesis has become a very promising target for cancer therapy. Advantages of targeting the growth of new blood vessels as opposed to other types of therapy include: the avoidance of tumor cell-resistance mechanisms, the broad and generally applicable mechanism, and easy access to targets within the vasculature. More than 20 endogenous inhibitors of angiogenesis and nearly as many stimulators that can serve as targets for small inhibitory molecules have been characterized (62). Interestingly, several standard chemotherapeutic drugs have been found to exert their antitumor effect in part through inhibition of angiogenesis.

There are over 75 antiangiogenic agents (proteins as well as small molecules) in clinical trials. In general, antitumor efficacy has been observed, although overall success rates have not lived up to expectations. One important reason is perhaps the choice of disease stage. Although antiangiogenic therapy will most likely be of highest efficacy at the early stages of tumor growth or metastasis, patients with excessive tumor burdens have been mostly treated so far.

Antiangiogenic gene therapy has not yet been tested in patients, but several preclinical experiments have shown promise (62). Among the first genes examined were the genes for the soluble form of endothelial cell receptor proteins, Tie2

and FLT-1. These proteins interact with the angiogenetic factors angiopoietin-1 and VEGF (vascular endothelial growth factor), respectively. These genes were transferred in vivo or ex vivo and resulted in significant inhibition of tumor growth (63–65). Other strategies include the transfer of antiangiogenic proteolytic fragments such as angiostatin or endostatin, of immunomodulatory genes with antiangiogenic properties such as interferons and IL-12, or of other molecules better known for other functions in the body such as tissue inhibitors of metalloproteinases (TIMPs), p53, or p16.

There are several advantages of molecular therapy over direct application of the effector substance. Firstly, the use of targeted vectors allows increased intratumoral concentrations of antiangiogenic factors without the risk of potential side effects on wound healing, endometrial maturation, or embryo growth (66). Secondly, the cost of gene delivery may be lower than prolonged protein therapy. Thirdly, experimental data suggest that effective antiangiogenic therapy requires the continuous presence of the inhibitor in the blood, which may perhaps be more efficiently achieved by gene therapy than by bolus protein therapy.

Antiangiogenic therapy itself is only in its infancy, and the future development of this treatment modality will obviously have a large impact on the corresponding gene therapy approach. Because of its mainly tumoristatic nature, it seems likely that this form of therapy will have its main impact before or after surgery, with a view to facilitating surgery and preventing recurrence of distant metastases, as well as in combination with conventional chemotherapy, radiotherapy, or immunotherapy.

Excessive degradation and remodeling of the extracellular matrix (ECM) is one of the hallmarks of cancer progression at nearly every step from the first breakdown of the basal membrane of a primary tumor up to the extended growth of established metastases. There are 4 known classes of proteases implicated in ECM turnover: matrix metalloproteinases (MMPs), serine proteinases, ADAMs, and the BMP1 family. The naturally occurring inhibitors of the MMPs, the 4 known TIMPs, and the inhibitors of serine proteases, the plasmin activator inhibitors (PAI), have been preclinically evaluated for antitumor efficacy and are the most prominent examples of antiproteolytic gene therapy. Intratumoral injection of first generation TIMP-2 adenovirus into subcutaneously growing tumors of mammary, colorectal, and bronchial origin reduced tumor size by 60–80% as compared to the controls, and the incidence of lung metastases was reduced by 90% (67). A similar efficacy was achieved by adenovirus-mediated transfer of TIMP-3 into subcutaneously growing melanomas (68). Systemic delivery of TIMP-2 by adenovirus application into the tail vein of immunocompetent mice bearing orthotopically implanted breast tumors resulted in 50% growth inhibition (69), and the intraperitoneal injection of TIMP-2 carrying liposomes for the treatment of spontaneously arising breast tumors allowed for a 40% size reduction and a dramatic reduction of the incidence of lung metastases (70). An intraperitoneal approach was taken to treat peritoneally disseminated pancreatic cancer, and adenoviral delivery of either TIMP-1 or TIMP-2 resulted in a 75% to 85% reduction of tumor loads and a significant prolongation of survival (71).

A prevention of colorectal liver metastasis of up to 95% was achieved if Ad-TIMP-2 was injected into the tail vein of mice, leading to a preferential transduction of the liver with the antitumor gene (72). Administration of TIMP-2 vector to mice bearing established liver metastases resulted in a 77% growth inhibition. When this same concept of gene transfer into the host tissue was applied for the treatment of lymphatic liver metastases, adenoviral transfer of TIMP-1 reduced metastatic deposits by 93% (73). The peculiarity of this approach is the transfer of anticancer defense genes into the normal noncancerous tissue, which was termed the ''impregnation'' approach.

In contrast to these findings of a marked antitumor efficacy of TIMP-1, -2, and -3 with 3 different types of animal models and modes of vector administration, systemic transfer of TIMP-4 by intramuscular electro-adjuvated injection of naked plasmid for the prevention of orthotopically growing breast tumors resulted in a 3-fold increase in size and up to a 7-fold increase in the incidence of primary tumors (74). None of the cited papers has reported any TIMP-related toxicity. Mild and probably vector-related toxicity to the liver was occasionally seen when first-generation adenovirus was delivered systemically.

Transfection of tumor cells with PAI-1, an inhibitor of serine proteases, reduced metastasis. Treatment with adenovirus-mediated PAI-1 gene transfer reduced the growth of uveal primary tumors as well as the incidence of metastases (75). Similarly, gene transfer of PAI-2 had unequivocal antitumoral effects (75). In contrast and unexpectedly, transgenic mice overexpressing PAI-1 developed pulmonary metastases at the same rate as wild-type mice in a melanoma model (76). Even more paradoxically, PAI-1 knock-out mice displayed a dramatically reduced local invasion and tumor vascularization of transplanted malignant keratinocytes and administration of PAI-1 by adenoviral gene transfer restored invasion and associated angiogenesis (77). These data indicate that antiproteolytic therapy with PAIs, and to a lesser extent with TIMPs, can have dramatic antitumor efficacy but that paradoxical tumor-promoting effects can occur. Similar to the MMPs and TIMPs, the PAs and PAIs have been shown to display effects independent from the degradation of the ECM (78), which may be partly responsible for the unexpected findings. Therefore, increased knowledge of either protease/inhibitor system may help us to better understand the underlying mechanisms, resolve some unexpected findings, and to draw conclusions with therapeutic relevance.

F. Drug-Resistance Genes

Instead of killing tumor cells, this strategy is aimed at preventing toxic side effects of modern chemotherapy, e.g., by making normal cells resistant to chemotherapeutic toxicity. This approach tries to solve the problem that the cytotoxicity of chemotherapy cannot usually discriminate between proliferat-

ing normal and cancer cells. Proliferating normal cells like hematopoietic precursor cells are especially affected, as evidenced by the fact that bone marrow suppression is still dose-limiting in most high-dose courses of chemotherapy. To overcome this limitation, different experimental approaches have been developed. One strategy to make hematopoietic precursor cells resistant to chemotherapy is the direct gene transfer of drug-resistance genes such as transporters that extrude drugs across the plasma membrane. These include the multiple drug-resistance gene 1 (MDR1), the multiple drug-resistance proteins (MRPs), or enzymes that render drugs resistant to alkylating agents such as gluthathion-S-transferase and O6-alkylguanine DNA alkyltransferase or antimetabolites such as dihydrofolate reductase (DHFR) (79). The MDR1 gene encodes for P-glycoprotein, a cell membrane multidrug transporter that effluxes a broad spectrum of hydrophobic and amphipathic compounds, including several chemotherapeutic drugs currently used in clinical studies (80). Ex vivo transfer and expression of the MDR1 gene in hematopoietic progenitor cells have been shown to increase the resistance of hematopoietic precursor cells to chemotherapy in rodent models (81–85). In the first of several clinical trials, low level expression of the transferred gene was reported only occasionally. More recently, however, prompt hematopoietic recovery and long-term in vivo vector expression was seen (86,87). We may see further clinical improvements if efficient transduction is achieved (especially of very immature cells) and drug selection strategies are further optimized. The encouraging report of in vivo correction of X-linked severe combined immunodeficiency (SCID) (88,89) with at least 5 of 5 patients now benefiting from this life-saving gene therapy also indicates that transduction of human hematopoietic stem cells is now feasible. Clinical benefit may result in situations where corrected cells have an in vivo selective advantage.

G. Marker Genes

Six percent of all clinical gene therapy protocols use marker genes for several purposes (90). Marker trials have confirmed the assumption that contamination of reinfused bone marrow with tumor cells causes tumor recurrence after autologous bone marrow transplantation (91). In other studies, hematopoietic stem cells or T-cells were marked and traced over a longer duration, providing important insights into their long-term survival.

III. VECTORS FOR CANCER GENE THERAPY

A. Virus-Based Gene Transfer Vectors

In this chapter, the various gene transfer vectors (e.g., adenovirus and retrovirus-based vectors) will be discussed with respect to their specific relevance for efficacy and toxicity in cancer gene therapy. For the general biology, development, and design of the vectors, the reader is referred to the corresponding chapters in this book that focus on particular vectors.

One of the most widely used vectors for in vivo application in cancer gene therapy is the replication-deficient recombinant adenovirus. First-generation adenoviral vectors (Ad vector) can be generated to the highest titers [up to 10^{12}/plaque forming (i.e., infectious) units/mL)] among viral vectors (92). Moreover, they easily infect cells of epithelial origin including cancer cells, most likely because these cells express the appropriate receptors, the coxsackie adenovirus receptor (93) and integrins (94) for binding and internalization, respectively. Since Ad vectors infect dividing and nondividing cells, dormant tumor cells, which can make up a considerable fraction of the tumor mass, can also be killed. These properties have made first-generation adenoviruses one of the vectors of choice for clinical studies targeting cancer. Of the 634 clinical gene therapy protocols initiated to date, 171 (26.9%) use Ad vectors, the majority of those for cancer treatment. Ad vector-mediated gene transfer can be accompanied by toxic side effects on normal tissue mainly due to residual adenoviral gene expression in occasionally transduced normal cells (95–100). This toxicity can be reduced using Ad vectors of the second generation where adenoviral genes such as the E4 region or the E2a gene are deleted (92,101–105), or helper-dependent (i.e., minimal or gutless) adenoviral vectors (HD-Ads), which are completely devoid of all viral genes (106–108). The side effects of first-generation Ad vectors such as toxicity and immunity can be turned into a desired adjuvant situation for cancer therapy as demonstrated by the group of Chen et al. (109). However, in cancer gene therapy where vector toxicity to the tumor cells at the first glance may be desirable, this toxicity may be of disadvantage in some situations. For instance, leakage of intratumorally injected vectors carrying a prodrug-converting enzyme (''suicide gene strategy'') into the host organ has been shown to produce severe side effects (20). Regional and systemic application of vectors with the aim of targeting metastases will inevitably transduce normal tissue. Other anticancer strategies are even explicitly based on transduction of normal tissue for its use as a bioreactor as in the antiproteolytic impregnation concept, antiangiogenic, and some immunological approaches. Therefore, cancer gene therapy (like gene therapy of other diseases) will depend enormously on safer vectors. In addition to increased safety (110), HD-Ads have some other advantages over first-generation vectors, including an extremely high packaging capacity that allows for a better adaptation to the tumor cell's particularities, as well as the potential for long-term gene expression [e.g., (111)], apparently higher gene expression (112), and better retained tumor-specific gene expression (113). The 2 main disadvantages of HD-Ads have been contamination with helper virus and difficulties in obtaining high titer preparations. Helper contamination has been significantly improved and titers of up to 2×10^{11} bfu (blue-forming units) are possible with modern techniques. HD-Ads have been tested in several preclinical models including cancer models (114), but no clinical trials have been conducted so far.

Another very popular vehicle for gene transfer, accounting for more than one-third of all trials and half of all patients treated, are retrovirus-based vectors. Most of these are derived

from murine leukemia virus (MLV). Retroviral vectors were the first vectors for which all the viral genes were provided by vector producer cells (VPCs). The retroviruses themselves contain only the LTRs, the packaging signal, and the transgene. In the field of cancer therapy, these vectors have dominated trials utilizing ex vivo transfer of markers, drug resistance, and immunomodulatory genes. For in vivo gene transfer, the VPCs themselves were often directly applied into the tumors due to the initially low achievable titers of 10^6/mL in the retroviral supernatant with the rationale that these VPCs will deliver retroviruses until they are eliminated by the immune system (115). As for adenoviral vectors, many animal studies were carried out, including in vivo gene transfer of retro VPCs or retroviral vectors into established tumors, and complete remissions of microscopic tumors have been reported [e.g., (115)]. Current packaging cell lines give titers of greater than 10^7 infectious particles per mL (116). Moreover, the use of different envelope proteins, such as the G protein from vesicular-stomatitis virus, has improved titers following concentration to greater than 10^9/mL (116), although these titers are still far below what is achievable with adenoviruses. Especially from a safety standpoint, their property of only infecting dividing cells may prevent toxicity to nondividing normal tissue. On the other hand, these vectors leave out dormant cancer cells, which may decrease their antitumor efficacy.

In contrast to MLV-based vectors, lentiviral retroviruses based on pseudotyped HIV-1 are able to infect nondividing cells (117–120), although there is some debate about whether hematopoetic stem cells should preferentially not be in the G_0 phase of the cell cycle (121) and whether hepatocytes are much more efficiently transduced in vivo if proliferating (122–124). Lentiviral vectors may therefore be of interest for cancer gene therapeutic approaches, which rely on long-term transduction of noncancerous tissue for cancer therapy. Mainly due to (perhaps overestimated) safety concerns with respect to the generation of wild-type virus, no clinical trials have been initiated yet. However, the use of apathogenic animal lentiviruses (125,126) may soon circumvent this concern.

Based on their tropism for neuronal tissues, herpes simplex viruses (HSVs) are in principle highly suitable for neurological cancer therapy. In general, they can infect dividing and nondividing cells. Similar to adenoviruses, cytotoxic genes must be deleted from the viral genome, and genes must be provided from helper viruses or cell lines in order to prevent unwanted toxicity if normal tissue is transduced. Successful attempts in this direction have been made (127–132). HSV has already been used in 5 clinical trials, and the intratumoral injection mode at least did not cause severe toxicity (see Section IV.D on Glioblastoma).

A fourth type of vector that has become very interesting for gene therapy in general during the last few years is adeno-associated virus (AAV)-based vectors. These helper virus (e.g., adenovirus)-dependent vectors induce only a minor cellular immune response and have the potential to integrate into the host genome. Therefore, they are very promising candidate vectors when long-term expression is required (for instance, for the protection of hematopoietic precursor cells during high-dose chemotherapy). With respect to clinical trials, AAVs are fairly advanced. Fifteen trials have already been initiated and at least 1 of them is planned for cancer (MediGene, Munich, German press release). For the general biology of AAV-based gene-transfer vectors, please refer to the respective chapter by Carter in this book (Chapter 5).

The poxviruses, fowlpox virus, and vaccinia virus infect a wide variety of cell types. In addition, they are safe and have a high packaging capacity (133). Due to their highly intrinsic immunogenicity, they have mainly been used as an adjuvant and transfer vehicle for immuno-gene therapy of cancer. This feature, as well as the nearly 100% preexisting antibodies, has prevented their more widespread use in gene therapy so far. Despite these limitations, an impressive number of 39 clinical studies using pox virus have been initiated.

In addition, several other viruses have been used for the transfer of genes into cancer cells. They usually have some interesting features but also bring certain disadvantages, which have so far prevented widespread use. EBV viruses have a natural tropism for B-cells, but the potential development of wild-type, transforming viruses by homologous recombination raises a safety concern. Baculoviruses are insect viruses and cannot replicate in human cells, but they have been shown to efficiently infect a variety of human cell lines (134,135). However, the human complement system rapidly inactivates these vectors such that extensive modification of the cell surface is required (135–137) to improve gene transfer in vivo. Alpha viruses such as Sindbis Virus (138) and Semliki Forest Virus (139) can efficiently multiply their RNA genome in target cells, allowing for very high transgene expression. Further improvements in their packaging systems could make these vectors very interesting agents for the future, especially for gene delivery to the brain, antitumor therapy by induction of apoptosis, or intratumoral replication and immuno-gene transfer (140).

Autonomous parvoviral vectors (141) such as H1, MVM, and LIII can only replicate during the S-Phase of the host cell and have been used as replication-competent viruses for cancer gene therapy in vitro or in preclinical settings (i.e., Newcastle disease virus, reovirus, poliovirus, and vesicular stomatitis virus) (142).

Chimeric vectors that are constructed by the use of 2 or more viruses may have the advantages and disadvantages of both vectors. To date, several types of hybrid vectors have been published, including first-generation adenovirus/AAV (143–145), HD-Ad/AAV (146,147), HD-Ad/transposon (148), HD-Ad/retrotransposon (149), first-generation adenovirus/retrovirus (150–152), or HD-Ad/lentivirus (153). These chimeras were evaluated mainly to combine the high efficacy of gene transfer with the long duration of gene expression; to facilitate AAV production; and to overcome the limited cloning capacity of AAV. HSV/AAV (154,155) chimeras or baculovirus/AAV chimeras (156) were constructed to combine cell specificity with stable gene expression.

With respect to long-term safety of viral vectors, occasional reports of possible oncogenicity have been described in animals for AAV (157) as well as for MLV-derived retroviruses (158), most likely due to insertional oncogenesis. This is not the case for HD-Ads, which can integrate at a low rate as well (159,160). Recently, a case of leukemia occurred in a trial for severe combined immunodeficiency that was very successful to that point.

Gene silencing as a reason for limited duration of gene expression despite vector integration has been described extensively for retrovirus and also for AAV (161), but, with the inclusion of MARs or SARs, matrix or scaffold attachment regions can improve the problem (162).

In summary, it seems that with respect to viral vectors it is possible to exploit nature's evolutionary achievements to our benefit and even to deviate markedly from nature's path without deleterious consequences. It is sometimes quite mysterious that even dramatic modifications of viral genomes still give viable and effective vectors. Since it seems that there is a way around nearly every problem, as long as time and money are provided, one can be confident that the vector problem—still the main issue in gene therapy—can be solved as long as the target diseases justify the effort and alternative ways of treatment are not provided.

B. Nonvirus-Based Gene Transfer Vectors

In general, nonviral vector systems have some advantages over viral systems. They are less toxic, less immunogenic, and easier to prepare. On the other hand, they have a much lower efficacy of gene transfer, particularly in vivo, and a more limited duration of gene expression than several viral vectors. Roughly 20% of clinical trials have utilized nonviral vectors. Half of these used naked DNA plasmids and the other half cationic liposomes, which are probably the most important nonviral vectors to date. Attempts to increase efficacy of gene transfer with cationic lipids and polymers will have to target 3 major barriers: stability during and after the manufacturing process, extracellular barriers, and intracellular barriers. An important step in improving extracorporeal stability of positively charged liposomes and polymers (which have a tendency to aggregate and precipitate) was the inclusion of hydrophilic polymers such as polyethylene glycol (PEG). This provides steric stabilization. PEGylation seems also to reduce interactions of cationic particles with blood components such as albumine, which present major extracellular barriers to nonviral gene transfer. Modifications to decrease unwanted preferential uptake of liposomes by the reticulo-endothelial system include the addition of sialic acid to make ''stealth'' liposomes (163). The most challenging and perhaps most rewarding task will be the elucidation of intracellular barriers to nonviral gene delivery. Cationic DNA complexes bind to the negatively charged cell membrane and are taken up by endocytosis. As for viral vectors, targeting by addition of ligands for cellular receptors has been used to circumvent extracellular barriers. For example, transferrin is a common ligand used to target tumor cells (164,165). Endocytosed DNA is largely retained in the endosomal compartment, and it is important that endosomal release is completed before lysosomal degradation initiates. Fusogenic lipids such as DOPE, fusogenic peptides from viral vectors (166), or substances with high buffering capacity (which in addition can swell like PEI) have been used to release DNA from endosomes. After vesicle escape, the nucleic acid must traffic to the nucleus. DNA is not very stable in the cytoplasm, which may be due to the presence of cytoplasmic nucleases. It is therefore particularly important to define the optimal time-point to release DNA from protection by the endosome or from coverage from vector after endosomal release. Once the vector arrives at the nucleus, another hurdle has to be overcome since nuclear entry of DNAs larger than 250 bp is hardly possible. A promising attempt to circumvent this problem is the inclusion of nuclear localization signals [NLS (167,168)].

Naked DNA is most interesting for immunological approaches in which efficacy of gene transfer is not the most crucial criterion. Interestingly, skeletal muscle cells are fairly susceptible to transfection with directly injected plasmid DNA. However, in mice, systemic application of naked DNA has led to astonishingly high rates of gene transfer in mice if administered systemically in extremely high volumes and with generation of high pressure in target organs (169,170).

In addition to liposomes and naked DNA, cationic polymers have gained attention during recent years, although no clinical trials yet exist. Other modalities include electroporation and particle bombardment (171–173). In this latter technique, 1–3 μm gold or tungsten particles are covered with plasmid DNA accelerated in an electrical field and fired onto the target tissue. Due to a penetration depth of up to 50 cell layers, this approach is applicable for vaccination into superficial skin cancers.

C. The Transgene

A gene therapeutic expression cassette consists of the regulatory elements, the transgene, and the poly A signal.

The transgene is usually a wild-type cDNA but other structures are possible. Among these are mutated transgenes, such as dominant negative oncogenes to inhibit oncogenes, or mutated cDNAs that lead to other defects favorable for cancer gene therapy. The availability of high-capacity vectors such as HD-Ad or HSV has allowed the inclusion of full-length genes, including introns and other features that may eventually be as interesting for cancer gene therapy as they already are for the treatment of monogenetic disease. Finally, recent developments of small, double-stranded DNA oligonucleotides that apparently can interfere with RNA may dramatically influence cancer gene therapy. These oligonucleotides have already been included in viral vectors, and antitumor efficacy has been reported after ex vivo gene transfer (39).

D. Cancer-specific Gene Expression (Transcriptional Targeting)

If one wants to exploit differences between tumor and normal tissue for tumor therapy, tumor-specific gene expression is a

particularly rewarding area. Transcriptional targeting restricts gene expression to the target tissue even if undesired tissues have been transduced as well, thereby providing a significant improvement in safety. Tumor-specific promoters are only active in those target tissues naturally expressing their respective tumor-specific endogenous genes. If one engineers an expression cassette in which any therapeutic gene is placed behind that tumor-specific promoter, it will, due to its promiscuous nature, express this transgene in a tumor-specific fashion as well. A huge variety of tumor-specific promoters have been examined during the last years for their use in gene therapy. This is basically because every tumor type has its own more or less tumor-specific genes. Tumor-specific gene expression can obviously only be as specific as the degree of differential gene expression of the respective endogenous gene. Therefore, it has certain limitations. In other words, residual gene expression in undesired tissues is frequently observed. Among the promoters used are tissue-specific promoters, which will also be active in the corresponding normal tissue, and proliferation-specific promoters, which will be active in proliferating nonmalignant tissue. In addition, the expression levels are often only gradually higher in the tumor than in the normal tissue. One must carefully examine how these gradual differences translate into the therapeutic situation.

Tumor-specific promoters are particularly interesting in the situation of metastatic cancer where the tissue-specific promoter allows transgene expression in the metastatic tumor cell but not in the surrounding normal tissue, even if it is transduced by the vector. For instance, the carcinoembryonic antigen (CEA), which is physiologically expressed predominantly in colon tissue but not in liver tissue, allows transgene expression predominantly in colon cancer cells metastatic to the liver. Toxicity to the liver seen with the ubiquitous CMV promoter that expresses a suicide gene (20,174) can be prevented if the CEA promoter is used instead (21). Other examples of such a promoter strategy include the AFP (alpha-fetoprotein) promoter for hepatocellular carcinoma (HCC); the promoters of erbB2, an oncogene often found in breast tumors; the prostate-specific antigen (PSA); or the promoter of the tyrosinase gene, which has specificity for melanoma.

However, the maintenance of tissue specificity can be a problem after inclusion into a vector. For adenoviral vectors, loss as well as maintenance of tissue specificity has been reported (21,175–180). In this context, the orientation of the expression cassette can play an important role. For instance, the transcriptional activity of the E1A enhancer, which is not deleted in E1-deficient first-generation adenoviruses, can induce loss of tissue specificity. This type of promoter interference can even stem from regions located downstream from the expression cassette (181). This can even occur if the expression cassette is inserted in the reverse orientation to the main adenoviral reading frame (author's observations). To circumvent this problem, an insulator element derived from the chicken gamma-globin locus (HS-4) was employed to shield an inducible promoter from viral enhancers. Induction ratios could be improved up to 40-fold as compared to vectors with an uninsulated promoter (181). In a similar approach, transcriptional terminators have been implemented to restrict read-through of the E1A enhancer (182). Finally, insertion of a specific promoter at a site distant from the E1A enhancer (e.g., close to the right ITR) has retained higher specificity and lower background (183).

In contrast to first-generation adenovirus, HD-Ads seem to allow full maintenance of promoter specificity. This has recently been demonstrated for the PSA and the tyrosinase promoters (113).

Also, in contrast to first-generation adenovirus, insertion of expression cassettes in reverse orientation into retroviral vectors normally maintains tissue specificity. Orientation in frame, however, puts the transgene under the influence of the strong retroviral LTR with subsequent loss of specificity (184). Alternatively, SIN (self-inactivating) vectors, which do not possess any promoter on the 3′ or 5′ end of their genome after integration into the host cell genome (185), can be used. The LTR can also be replaced by the tissue-specific promoter. The problems of tissue-specific expression seen in adenovirus or retrovirus vectors do not seem to occur in AAV vectors (186,187). This is probably because the flanking ITRs possess no regulative activity. Recently, maintenance of tissue-specific expression was reported for the PSA promoter in a lentiviral vector (188).

A major problem with tumor-specific promoters, whose specificity may be satisfying, can be insufficient promoter strength. An ingenious strategy to compensate for the weakness of tumor-specific promoters has been made possible through the introduction of the CRE lox system into the concept of transcriptional targeting. On 1 of the constructs, the tumor-specific promoter regulates the expression of the CRE recombinase. This enzyme then recognizes loxP sites (on a second construct), which flank a spacer region separating a strong viral promoter and a therapeutic transgene. Cleavage of the loxP sites excises the spacer, thereby inducing transcription of the transgene. Expression of the transgene with this system is supposed to be higher than with simple tumor-specific expression because the stability of the CRE enzyme will amplify the tumor-specific effect. The utility of this system has been proven using the AFP promoter in vitro (189) and the CEA promoter in vivo (190). Other strategies to compensate for promoter weakness have used transcriptional transactivators under tissue-specific control, which then have activated a minimal promoter for transgene expression (191–193). So far, all these systems have used 2 types of adenoviruses for either promoter/transgene construct. However, incorporation of both expression cassettes on 1 vector should be possible.

As in other fields of gene therapy, the packaging size of the vector to be used for gene transfer is one of the most important limitations for the construction of cancer-specific expression cassettes. So far, vectorologists have mainly worked with the intron-free cDNA coding for the transgene. Recently, the availability of vectors with substantially increased packaging capacity, such as HD adenoviruses, has allowed the inclusion of whole mini-genes into a viral vector

(107,144,194,195). This provides the necessary space for longer and potentially even more specific regulatory elements.

In contrast to constitutive promoters, tumor specificity in patients who are resistant to chemotherapy could be mediated by therapy-inducible promoters. In this respect, the examination of gene-regulation mechanisms in cancer cells such as the multidrug resistance gene (MDR1), the X-irradiation-induced, tissue-type, plasminogen activator (t-PA), the early growth response gene (Egr-1), the human, heat-shock protein HSP 70, or the glucose-regulated protein (GRP78) led to the discovery of a class of promoter sequences that are involved in such stress responses. These promoters carry responsive elements that are inducible by either radiotherapy, cytostatic drugs, or hyperthermia (all conventional treatment modalities). It has already been shown that the expression of therapeutic genes could be enhanced and the efficacy increased if placed under the control of therapy-inducible promoters (196). The combination of therapeutic genes under the control of therapy-inducible promoters used with conventional cancer treatment methods could enhance overall treatment efficacy and also retain specificity.

E. Targeted Vectors

Currently, the predominantly used viral vectors for gene transfer can infect a broad variety of target cells and tissues. Although this is of interest for cell-type, independent, gene expression, it is a disadvantage when tissue-specific, gene expression is required as in cancer gene therapy. Therefore, the surface of the vector needs to be modified to infect the cancer cell in a more specific manner (retargeting).

Several approaches have been developed to redirect the tropism of adenoviral vectors in favor of cancer cells. The efficacy of binding to the surface of a given target cell by an adenovirus depends on the presence of specific receptors such as the coxsackie-adenovirus receptor (CAR) and to a lesser extent the alpha2 domain of MHC class I on the cell surface, which interact with the adenoviral fiber knob protein (93,94,197,198). After binding, the adenovirus is internalized through clathrin-coated pits. This process is mediated through an interaction between RGD motifs on adenoviral penton base protein loops and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Two major strategies have been used to redirect adenoviral infection: conjugate-based strategies and genetic targeting strategies. Conjugate-based strategies include bispecific recombinant proteins, peptides, or chemical conjugates to redirect adenoviral infection. Examples include bispecific conjugates consisting of the Fab fragment of a neutralizing antiknob monoclonal antibody covalently linked to folate (199–201); polymer-mediated ligand coupled to adenoviral capsid, or avidin bridging between biotinylated adenovirus; and biotinylated potential ligands for cellular receptors. Increases of 100-fold and more in infectability have been described in several in vitro studies as compared to wild-type adenovirus. The best results reported so far for in vivo systemic vascular delivery of 2-component, targeted vectors were obtained using adenovirus with bispecific antibodies. These blocked the CAR binding domain and redirected the vector either to: FGF receptors on tumor cells, which led to substantially increased survival using the HSVtk/GCV approach (202), or to angiotensin-converting enzyme on pulmonary capillary epithelium, which enhanced gene transfer to the lung at least 20-fold. The same group has recently initiated the first clinical study using retargeted adenoviruses for the treatment of cancer with a suicide gene approach (203).

Conjugate-based approaches have 2 main advantages: the wide range of cellular receptors that can be targeted and the fact that there is no need to make structural changes to the adenovirus itself. The disadvantages of the system include the larger size and irregular shape of the particles, as well as regulatory issues such as the need to obtain approval of all components of a 2- or 3-component system.

These problems can be circumvented by genetic targeting approaches that genetically modify fiber, penton base, and hexon capsid proteins. By far the most extensively studied alterations have been done on fiber knob, including fiber knob pseudotyping, genetic incorporation of targeting ligands into the fiber protein, and genetic fiber replacement strategies. Fortunately, 2 sites in the fiber protein have been identified where ligands can be inserted safely without disrupting fiber-knob trimerization. These are the C-terminal end of the fiber tail, which projects from the surface of the virion, and the so-called HI loop within the fiber knob. Incorporation of targeting ligands led to increased infectabilitiy of target cells in vitro of up to 3 orders of magnitude (204,205). The advantage of entire replacement of fiber over just incorporating ligands is the possibility of achieving real retargeting, because only then can initial affinity of adenovirus to CAR be abrogated. To date, attempts have been successfully made to abrogate infection of CAR positive cells, but infectability of retargeted adenoviruses in general was lowered 20-fold (206,207). A pseudotyped adenoviral vector has recently been utilized for targeting replication-competent adenovirus to liver metastases, and an increase of the transduction rate from 0% to 8% was observed (208).

A combination of conjugate-based strategies and genetic targeting has recently been described. Efficient and selective gene transfer into human brain tumor spheroids has been achieved using antibody-targeted vectors; native tropism to both CAR and integrin were abolished (209).

A variety of retargeting attempts have recently been undertaken for AAV. The primary attachment receptor for AAV is heparan sulphate proteoglycan (210), and coreceptors are fibroblast growth factor receptor-1 (211) and $\alpha_v\beta_5$ integrins (212). These receptors are widely expressed in the human body. Retargeting is therefore feasible, especially as AAV is an integrating vector. In addition, it seems that despite efficient internalization, nuclear trafficking of AAV may be inefficient. Retargeting to new receptors could allow for new and more efficient ways of trafficking. As for adenoviruses, genetic and nongenetic approaches have been used to retarget AAV. The former strategy has been more popular by far, however. In vivo gene transfer has been performed with AAV-2 pseudotyped with AAV-5 capsid or AAV-8 capsid; this pro-

duced higher levels of transduction than wild-type AAV-2 in injected skeletal mouse muscle or liver, respectively (213).

Much effort has recently been made in genetically incorporating targeting ligands into the AAV capsid. In contrast to adenovirus, the structure of the AAV capsid has not yet been elucidated. This will eventually enable pinpointing of the capsid epitopes, which mediate cell-surface, receptor binding and enable specific site-directed mutagenesis for ablation of wild-type AAV-2 tropism. Despite this drawback, by modeling the AAV capsid on the known structure of canine parvovirus, several studies have already demonstrated that genetic incorporation of peptide epitopes into the AAV capsid can modify tropism.

The first genetically targeted HD-Ad has recently been published (214).

The wild types of commonly used retroviruses such as ecotropic murine leukemia virus (MLV) strains do not recognize a receptor on human cells. They have therefore been pseudotyped with natural viral fusion proteins such as the amphotrophic MLV env protein or the vesicular stomatitis virus (VSV)-G glycoproteins (215). These pseudotyped retroviruses now infect a variety of human cells. This is not always desirable, as in the case of adenovirus and AAV. Therefore, retargeting of these vectors to specific cell types has been attempted. In addition, studies have shown that several cell types, including hematopoietic stem cells, are still insufficiently infected by pseudotyped vectors. Some specificity is achieved just by using other viruses for pseudotyping (such as gibbon ape leukemia virus (GALV) (216), human foamy virus (HFV) (217), simian immunodeficiency virus (218), and HIV-1 (219)).

More was expected from direct targeting approaches such as inclusion of a wide variety of ligands into the MLV glycoproteins. Efficient retargeting was achieved in several cases. Unfortunately, upon binding most of the chimeras were unable to trigger the highly complicated fusion events of viral envelope and target cell membrane necessary for efficient viral transduction. The most straightforward approach to circumvent this problem was the creation of tethering or escorting ligands. These were again cloned into the MLV env glycoproteins, but at a site that does not abrogate wild-type receptor interaction and disturb fusion. This type of retargeting has already proven successful for preclinical cancer therapy. Systemic application of an extracellular, matrix-targeted retrovirus allowed enhanced transduction of tumor tissue and significantly prolonged survival of mice as compared to a nontargeted vector (220). During these studies, an observation was made that was paradoxical at first glance: some receptor ligands conferred excellent binding to target cells, but due to lysosomal sequestration no gene transfer was achieved (221–223). This phenomenon, which has been called inverse targeting, can cause the selective transduction of EGF-receptor negative tissue if the vector has been engineered to contain EGF and if the EGF-receptor negative tissue is principally infectable by the wild-type vector (as has been demonstrated by inverse targeting of lentiviral vectors to the spleen) (224). This type of inverse targeting can be reversed into true targeting by inclusion of a protease cleavage site inserted between the EGF domain and the amphotrophic receptor. In this case, EGF serves as a tethering molecule, but extracellular proteases present on the target cells cleave it off and efficient infection occurs. This approach has proven to discriminate between (matrix metallo) protease rich and poor in EGF receptor-positive tumors in nude mice (225).

Another innovative way to achieve retargeting without losing fusion uses proteins of nonretroviral, enveloped viruses instead of the natural receptors. These proteins are able to trigger membrane fusion. MLV vectors coated with the influenza virus hemagglutinin (HA) glycoprotein, including a cell surface-specific ligand, could be selectively targeted to and incorporated into cells expressing the expected target cell surface molecules (226). Finally, similarly to adenovirus and AAV, bifunctional bridge proteins have been examined for targeting with success in vitro (227).

The benefit of combining transcriptional targeting and tropism-modified targeting was recently demonstrated using the endothelial, cell-specific FLT-1 promoter and an antiACE antibody. This combination achieved an impressive 300,000-fold improvement in adenoviral transduction of the lung (228).

IV. CLINICAL TARGETS FOR CANCER GENE THERAPY

A. Bladder Cancer

Superficial transitional cell cancer of the bladder (TCC) is the fifth most common solid malignancy in the U.S. (229). Seventy percent to 80% of patients with bladder cancer present with these low-grade, noninvasive tumors confined to mucosa. Although most superficial cancers can be managed with periodic transurethral resection, this is not an ideal situation because recurrence is the rule (230). Even with close surveillance and follow-up, at least 50% will eventually require a cystectomy. Intravesical chemotherapy or installation of Bacille Calmette-Guérin can prolong the disease, but still 30% of patients will die of recurrent metastatic bladder cancer within 15 years (230).

Intravesical gene therapy is a promising new approach for the treatment of refractory superficial bladder cancer. Several approaches have proven preclinical efficacy, including transfer of immunomodulatory genes such as IL-2 (231) and IFN-γ (232); installation of tumor cells transfected with GM-CSF, IL-2, or HLA B7-1; HSVtk-based suicide approaches (28,29); oncolytic Herpes virus, which was interestingly efficient against metastases if injected systemically (32); and transfer of p16 (35), pRb (33), or p53 (13).

A clinical trial of installation of empty vaccinia virus did not lead to any systemic toxicity, but recruitment of lymphocytes and induction of a brisk local inflammatory response were seen (233). Intravesical installation (but not intratumoral injection) of Adeno-p53 led to transgene expression and induction of the p53 target gene p21 in patients with bladder cancer awaiting total bladder resection. No dose-limiting toxicity was observed at a dose of up to 7.5×10^{13} particles (234).

The superficial nature of TCC and the unique opportunity of intravesical vector installation make this disease particularly suited for cancer gene therapy at the current state of development.

B. Breast Cancer

One of the most important target malignancies for cancer gene therapy is breast cancer. Breast cancer will affect 1 in every 9 women in the United States, and a similar incidence is seen in Europe (235). Conventional treatments such as surgery, radiotherapy, and adjuvant systemic therapy allow disease-free survival for many years. However, the loco regional recurrence rate and the rate of disseminated disease is high, and even 10–40% of patients without obvious axillary lymph node involvement at the time of surgery relapse (236). In these cases a curability by conventional methods is very unlikely.

Based on the unsatisfying outcome of classical strategies to improve cancer treatment, several gene-transfer approaches are under experimental and clinical investigation. These include immunological approaches or the transfer of tumor-suppressor genes or prodrug-activating enzymes.

Several immunological strategies are based on the use of tumor-specific antigens to improve recognition of breast cancer cells by the effector cells of the immune system. In this respect, several tumor-specific antigens are under experimental investigation to set up a specific vaccination strategy for patients with breast cancer. Candidate antigens are mucin 1 (MUC-1), MAGE-1, carcinoembryonal antigen (CEA), and members of the erbB gene family of cell surface receptors (237). A recombinant vaccinia virus expressing MUC1 and IL-2 has been used in a phase I/II study in 9 patients with advanced breast cancer (238); 1 patient had a concomitant decrease in carcinoembryonic antigen serum levels and remained clinically stable for 10 weeks.

Among the first immunological approaches for breast cancer patients was a phase-I study of immunotherapy of cutaneous metastasis using allogenic (A2, HLA-B13) and xenogenic [HLA-H-2K(k)] MHC-DNA liposome complexes (239). Several partial and complete responses of the injected tumorous nodules were observed.

Intra-tumoral adenovirus-mediated gene transfer of the 4-1BB costimulatory molecule-ligand (ADV/4-1BBL) to liver metastases in a syngeneic animal model of breast cancer induced a dramatic regression of preestablished tumor (240). Excellent tumor regressions were also observed with a combination of IL-2 and IL-12-expressing adenovirus in a mouse model of mammary adenocarcinoma; 63% of animals underwent complete regression of both treated and untreated tumors (241). Direct in vivo IFN-β gene delivery into established tumors generated high local concentrations of IFN-β, inhibited tumor growth, and in many cases caused complete tumor regression. Because the mice were immune-deficient, it is likely that the antitumor effect was exerted primarily through direct interferon-mediated inhibition of tumor cell proliferation and survival (242).

Gene transfer strategies for breast cancer patients can also be used to improve classical strategies in breast cancer therapy such as chemotherapy. In this field, purging techniques in high-dose chemotherapy are under intensive experimental investigation. Before initiation of high-dose chemotherapy for women with breast cancer, autologous stem-cell transplants are collected from the patient and are given back after the therapeutic protocol is completed. Because these preparations can still be contaminated by tumor cells, different strategies aim at purging these stem-cell transplants from contamination. However, magnetic purging techniques are expensive and not very efficient, while pharmacological purging is very effective but toxic not only to the tumor (243). Therefore, to limit the toxicity of pharmacological purging of contaminating cancer cells, either hematopoietic precursor cells of the autologous stem-cell transplants need to be made resistant to chemotherapy, or contaminating tumor cells need to be made more sensitive to chemotherapy. Thus, one genetic approach tries to infect only hematopoietic cells and make them resistant to chemotherapy through transfer of a multidrug-resistance gene (244). An example of the other strategy of making contaminating tumor cells more sensitive to chemotherapy is adenovirus-mediated gene transfer of a prodrug-activating enzyme like HSV-tk or CD (175,245,246). This approach exploits the fact that breast cancer cells that contaminate autologous stem-cell transplants express more adenovirus-internalizing integrins than hematopoietic precursor cells (247–249). Preclinical studies have already demonstrated that the concept of adenovirus-mediated gene transfer into contaminating breast cancer cells of autologous stem cells is a real alternative strategy for direct clinical application (250,251). Purging of hematopoietic stem cells from breast cancer cells has also been successfully performed with conditionally replication-competent HSV (252), and a complete lack of toxicity has been shown for a p53 adenovirus (253).

Another strategy that has been adapted for gene therapy of breast cancer is the use of antioncogene and tumor suppressor genes. In vivo administration of BRCA1 led to tumor growth retardation comparable to that obtained with p53 or p21 (254).

Hydrodynamic-based gene delivery of a secreted form of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand, TRAIL, led to the regression of a human breast tumor established in SCID mice (255).

Adenovirus-mediated transfer of the cDNA of the proapoptotic bcl-x_s into breast cancer cells led to a significant reduction of tumor growth after the transduced cells were transplanted into immunodeficient mice (24,256). As described in the literature on colorectal cancer, breast cancer also develops by a succession of genetic alterations (257–261). Although the degree of genetic heterogeneity is particularly high in breast cancer, these multiple genetic changes may interfere with just a few critical cell-cycle regulatory pathways and therefore represent suitable targets for corrective gene therapy (262). Successful in vitro approaches include oligonucleotide-mediated transfer of antisense myc (263,264), ErbB-2 (265,266), cyclin D1 (267), and TGF alpha (268); the transfer of genes for intracellular antibodies, which prevent growth

factor receptors to reach the cell surface, as reported for ErbB-2 (269); and other transgenes such as PKA, EGFR, TGF-beta, IGFIR, P12, MDM2, BRCA, Bcl-2, ER, VEGF, MDR, ferritin, transferrin receptor, IRE, C-fos, HSP27, C-myc, C-raf, and metallothionin genes (270). Some of these have already been tested in vivo. For example, excellent inhibition of breast tumor growth in vivo was shown by retroviral transfer of antisense C-FOS (271).

The best existing in vivo data so far has been seen with adenoviral transfer of the tumor suppressor p53, which argues for the fruitfulness of combining a highly efficient gene transfer vehicle with a nearly universal apoptosis inducer (272). In addition, good efficacy of tumor-growth reduction was achieved using liposomes for the transfer of a p53 cDNA (273). The importance of the p53 status for the efficacy of chemotherapeutic drugs is a matter of intense debate. Whereas in the majority of studies the loss of p53 has been associated with decreased sensitivity to chemotherapy, the opposite was reported for the chemotherapeutic drug taxol (274). An interesting approach to counteract the MDM2 protein, which is often elevated in wtp53 positive tumors, was conducted by generating a recombinant adenovirus expressing a p53 variant (that is, deleted for the amino acid sequence necessary for MDM2 binding). Apoptotic activity of rAd-p53 expressing the mutated p53 with that of a recombinant adenovirus expressing wild-type p53 in cell lines that differed in endogenous p53 status caused higher levels of apoptosis in p53 wild-type tumor lines compared with wild-type p53 treatment, showed apoptotic activity similar to that seen with wild-type p53 treatment in p53-altered tumor lines, and showed greater antitumor activity in an established p53 wild-type (hepatocellular) tumor compared with treatment with wild-type p53 (275). With a similarly mutated adenovirus, tumor regressions were also observed in a sarcoma model (276). In combination with chemotherapy, coapplication of docorubicin did show an overadditive effect (277), whereas cyclophosphamide did not (278).

Stable preclinical tumor regressions were also achieved by intratumoral injection of an adenovirus harboring the adenoviral E1A gene under the control of the tumor-specific MUC1 promoter with and without an additional TNF-a transgene expression cassette (52). In a clinical trial, an E1A gene complexed with DCC-E1A cationic liposome was injected once a week into the thoracic or peritoneal cavity of 18 patients with advanced cancer of the breast ($n = 6$) or ovary ($n = 12$) and led to HER-2/neu downregulation, increased apoptosis, and reduced proliferation (279).

Injection of a plasmid harboring the CD gene under the control of the tumor-specific erbB-2 promoter in 12 patients with breast cancer showed the safety of the approach; expression of the transgene was seen in 90% of cases, although no antitumor efficacy was reported (280).

Injection of adenovirus-harboring dominant negative mutants of estrogen receptors into preestablished T47D tumors in nude mice induced tumor regressions (281).

Finally, in vivo studies of reovirus breast cancer therapy revealed that viral administration could cause tumor regression in an MDA-MB-435S mammary fat pad model in severe combined immunodeficient mice. Reovirus could also affect regression of tumors remote from the injection site in an MDA-MB-468 bilateral tumor model, raising the possibility of systemic therapy of breast cancer by the oncolytic agent (282).

In summary, although metastatic breast cancer poses high demands on current cancer gene therapists, nearly all therapeutic principles have been evaluated in animals and some are already in clinical evaluation.

C. Colorectal Cancer

Colorectal cancer is the third most frequent cancer in the United States (283). Surgical removal of the primary tumor is the established first-line therapy. This strategy can be a curative approach if all cancer cells are eliminated. However, already at a very early stage of tumor development, colorectal cancer metastasizes to the liver. In 60% of all cases, the only manifestation of distant metastasis (284) occurs in this organ. Consequently, liver metastasis of colorectal cancer is one of the very few indications where treatment of metastasis can lead to a significant improvement of the prognosis of the disease.

Single-liver metastasis of colorectal cancer represents a promising target for intratumoral and/or regional gene therapy. Studies in experimental animals demonstrated significant tumor reduction by gene transfer of the tumor suppressor gene p53 (285). However, neither a clinical trial applying p53 adenovirus into the hepatic artery (286,287) nor intratumoral injections (288) have revealed any clinical response. The suicide approach by gene transfer of prodrug-activating enzymes such as cytosine deaminase, HSV-tk, or nitroreductase followed by systemic application of the corresponding prodrugs 5-fluorocytosine, ganciclovir (GCV), or CB1954 has led to good results in animal experiments (289–291). A single dose of adenovirus transferring the HSV-tk gene, followed by a 10-day intraperitoneal GCV treatment, led to a reduced tumor growth of more than 90% and a significant reduction of the tumor volume (292,293). This experimental approach was extended by the combination of adenovirus vectors carrying the transgene of different cytokines with the adenovirus encoding for HSV-tk. The results so far have suggested that the HSV-tk/GCV effect can be increased by simultaneous cytokine gene expression (292). However, in an orthotopic model of colon carcinoma metastatic to the liver, the HSV-tk strategy was compromised by severe hepatic toxicity and the death of several animals (20). Similar toxicity was seen if the vector was applied intraportally for the treatment of HCC in mice or rats (174). In contrast, no toxicity was observed with retroviral vectors (294) (author's findings). The liver toxicity observed using adenovirus vectors could be abrogated if the CMV promoter was replaced by the colon-specific CEA promoter in these vectors (21). The generation of tumor-cell-specific transgene expression in colorectal liver metastases by adenovirus vectors can also be accomplished by modification of the adenoviral fiber protein by inclusion of the CEA receptor (295). Recently, the results of an adeno-HSVtk/GCV phase I

trial with intratumoral injection of an RSV.HSVtk-adenovirus were reported. Ultrasound-guided percutaneous injection of up to 10^{13} adenoviral vector particles only caused mild hepatic toxicity upon GCV treatment; this may indicate that extensive leakage into the liver tissue was prevented. No clinical benefit was observed (296).

A clinical phase I trial with conditionally oncolytic, replication-competent, E1B55K-deleted adenovirus was performed (297). It was possible to safely deliver virus by direct intratumoral, intra-arterial, and intravenous injection up to a dose of 3×10^{11} infectious particles. In a phase II trial for patients with colorectal liver metastases, virus was administered intra-arterially together with the chemotherapeutic 5-FU. After 3 months, stable disease was demonstrated in 6 of 7 patients (297).

Antiangiogenic and antiproteolytic strategies have been implemented but are still in the preclinical phase. A prevention of colorectal liver metastasis of up to 95% was achieved when Ad-TIMP-2 was injected into the tail vein of mice; this leads to a preferential transduction and quasi-impregnation of the liver with the antitumor gene (72).

Multiple liver metastases are also targets for immunological gene therapy strategies. In this respect, the CEA is one of the most promising candidates under investigation. In 2 clinical studies of recombinant vaccinia virus-CEA immunization, T-cell responses were demonstrated (298,299). In a recently published clinical trial of a dual-expression, plasmid-encoding, carcinoembryonic antigen (CEA) and hepatitis B surface antigen (HBsAg) in 17 patients with metastatic colorectal carcinoma, 4 patients developed lymphoproliferative responses to CEA after vaccination, although no objective clinical responses to the DNA vaccine were observed (300). In addition, significant tumor growth inhibition of xenografts from colon tumor cells in established animal models has also been accomplished using different approaches. These include: vaccinia virus-mediated transfer of B7-1 and IL-12 (301); adenovirus-mediated transfer of IL-12 (294); liposomal transfer of MHC class I molecules (302); or the transfer of fibroblasts that had been transduced in vitro with IL-2 (303) (this last result has led to the initiation of a clinical phase I study) (304). The results of these experimental studies indicate that the dormant or suppressed immunogenicity of colon tumor cells can be evoked by several immunomodulatory mechanisms. Moreover, the good antitumor efficacy achieved even with vector systems, which traditionally suffer from low gene-transfer efficacy, suggests that a certain level of gene transfer may be sufficient to induce an immune response.

In summary, colorectal liver metastases are unique. Their cure could mean a cure for the patient. Therefore, they have served as the model disease for gene therapy of metastatic disease. Initial, encouraging clinical results have been obtained.

D. Glioblastoma

Glioblastoma is the most common primary brain tumor. Despite advances in diagnosis and treatment, the median survival time is still only 1 year from the time of diagnosis (305). This tumor rarely metastasizes to distant organs, suggesting that improvements in local treatment could be of great benefit. Therefore, glioblastoma has been one of the model diseases for gene therapy with suicide genes. Recent reviews on this topic are provided by (306,307). The first preclinical studies for cancer gene therapy were performed using retrovirus-mediated transfer of the HSV-tk gene into established intracranial glioblastomas in Fisher rats (115). Based on these early promising results, other vector systems have been tested for the HSV-tk/GCV approach including adenoviruses, liposomes, AAV, or HSV. All these vectors have been successfully used in animal studies where it was frequently possible to observe complete remissions with long-term survival (Table 1). Consequently, several clinical phase I, II, and III trials were initiated making the gene therapy of glioblastoma by the HSV-tk/GSV approach the most advanced system for cancer gene therapy. The suicide gene approach has been combined with surgery. As much malignant tissue as possible is removed, leaving the local infiltrating parts for multiple vector injections. This strategy has to leave out the large isles of healthy tissue within the tumor network, which is a characteristic of this tumor. This is the reason why complete resections can be rarely performed. As demonstrated by results from a multicenter phase II trial in Germany, there is no ''clear-cut'' clinical outcome so far. In a 1-year follow-up report of 10 patients with recurrent glioblastoma multiforme, where retroviral vector packaging cells were administered into the tumor followed by application of GCV, 4 of the 10 patients died because of tumor progression. Of the other 6 patients, 1 presented a complete remission at 12 months, and 5 had progressive disease but with a significant increase in quality of life (308). Other reports demonstrated responses in the CT scan; a clear enhancement was visible in the areas were the retroviral VPCs carrying the transgene had been injected (309). However, clinical responses were rare and not marked. Unfortunately, the first phase III trial with HSV-tk retroVPCs did not have any treatment benefit (310). Some responders were observed in trials using adenoviral vectors, but therapy was accompanied by severe neurological symptoms (311,312). As discussed in the context of colorectal liver metastases, toxicity can in principle be abrogated by tissue-specific expression of the transgene e.g., by the nestin (313) or myelin basic protein (25) promoter or targeted vectors (310). Three phase I trials with conditionally replication-competent HSVs have been performed to date. Toxicities related to the vector were not reported, and occasionally antitumor efficacy was seen (314–316).

Besides the strategy of using prodrug-converting enzymes, gene transfer of the tumor suppressor gene p53 has also been commonly tested in experimental models with success either alone (317,318) or in combination with radiotherapy and chemotherapy (319–321). Among the cell-cycle inhibitory proteins, p16 is very often inactivated in glioblastoma. Adenovirus-mediated gene transfer of p16 generated significant tumor growth reduction in p16-negative glioblastomas (322). For the transcription factor E2F-1, it could be demonstrated that adenovirus-mediated overexpression resulted in a tumor

Table 1 Selected Recent Reviews and Books on Some of the Covered Topics

Topic	References
Cancer gene therapy in general	[46, 465]
Oncolytic viruses	[142]
Angiogenesis	[62]
Antiproteolysis	[466]
Nonviral vectors general	[467–469]
Cationic polymers	[470]
Targeting:	
Adenovirus	[203–205]
Retrovirus	[471]
AAV	[205]
Lentivirus	[205]
Cancer types:	
Bladder	[472]
Breast	[473]
Brain	[307, 474]
Colon	[286, 475]
Head and neck	[476]
Hematological	[426, 477–479]
Lung	[393, 480]
Melanoma	[481]
Ovary	[482]
Pancreas	[438, 483]
Prostate	[484]

growth reduction in p53 wild-type-expressing glioblastoma cells (323). Other apoptosis-inducing genes [caspases (324) (325), TNF-α (326,327)], or antioncogenes [ras, (328), meningioma] have been successfully evaluated in animal models. In addition to the use of molecules regulating cell cycle and apoptosis, several immunomodulatory genes have been tested to treat experimental glioblastoma tumors either alone or in combination (329–332). Growth inhibition was seen in most cases, which indicates that although the brain is an ''immuno privileged'' site, this barrier could effectively be surmounted at least in some tumors. A clinical trial using interferon-β (333) has been initiated.

In summary, although the HSV-tk suicide gene trials probably represented one of the earliest sobering results in gene therapy, the disease is certainly a very good candidate for gene therapy. This is especially so if targeted vectors will distinguish between normal brain tissue and the deeply infiltrating and surgically inaccessible tumor branches of glioblastoma multiforme.

E. Head and Neck Cancer

Each year in the U.S., approximately 40,000 individuals will be diagnosed with carcinoma of the head and neck (HNSCC) and upper aerodigestive tract (334). More than two-thirds of the individuals with HNSCC present with stage II or IV of the disease (335) and 50–60% of these patients will ultimately develop local recurrence despite optimal local therapy. These patients may therefore obtain significant benefit from local or regional gene therapeutic approaches.

A high percentage of these tumors is negative for p53, and several clinical trials with percutanous or endobronchial p53 gene transfer are underway or already closed. Several clinical studies have used adenoviral vectors, while one trial used retroviruses. In summary, no major toxicity was observed. Indications of antitumor efficacy included increased survival times compared to historical controls in 4 of 15 patients with recurrent disease; dose-related improvements in time to disease progression in a large phase II trial; and partial response or at least stable disease in two-thirds of patients in a trial with 52 patients who had previously failed conventional treatment.

Despite these findings of clinical efficacy, improvements of the strategy are necessary, mainly because of the requirement of highly efficient gene transfer. Based on the bulky mass of head and neck cancer, it is unlikely that the large tumor burdens, which remain even after radical surgery, can be sufficiently transduced even by the highly efficacious, replication-deficient adenoviral vectors. Therefore, and because of the good accessibility, head and neck cancer has become the model disease for therapy with selectively replication-competent adenoviruses. The underlying concept of the use of replication-competent recombinant adenoviruses is based on the capability of adenovirus to induce cell lysis by its progeny inside an infected target cell (336,337). To limit the cytotoxicity only to tumor cells and not to normal cells, researchers exploited the fact that about 50% of tumors carry a mutation in the tumor suppressor gene p53. A recombinant, replication-competent adenovirus was constructed that should specifically replicate only in p53-negative tumor cells (43). For further explanation of this concept, please see Chapter II.D. Although the concept underlying the mode of action of the vector is not yet completely understood, several clinical trials have been performed. The published data, mainly with the vector ONYX-015, are among the most encouraging in clinical cancer gene therapy. With respect to toxicity, results suggest that the intratumoral injection of ONYX-15 limits proliferation of the vector to malignant tissue; it is well tolerated except for transient low-grade fever and injection site pain. Three clinical trials have been formally published so far (338–340): a phase I trial, a phase II trial for intratumoral injection of ONYX-15 alone, and 1 trial for the combination of virus and cisplatin. Five of 36 patients in the phase II trial showed partial or even complete ($n = 3$) responses (339). In the combinatorial approach, 63% as compared to 30–40% in the historical controls showed partial or complete responses (340).

Besides these dominant strategies for HNSCC, immune modulatory approaches have already been tested clinically. Partial responses have been observed in 4 out of 9 patients with advanced HNSCC upon intratumoral injection of HLA-B7 plasmid (Allovectin-7) (94). Among several approaches showing promising preclinical efficacy, including transfer of suicide genes, Bcl-2, superoxide, dismutase (SOD), and EGF receptor, transfer of adenovirus E1A in a liposomal formula-

tion has been tested clinically. It showed only minimal toxicity and modest clinical efficacy with 1 out of 24 patients with recurrent, unresectable HNSCC having a complete response; 2 patients having a minor response; and 7 patients having stable disease (341).

Head and neck cancer is probably the tumor type with the best clinical results in cancer gene therapy obtained so far. This may be due to the good accessibility for intratumoral approaches; the low tendency for metastazation, with the opportunity for long-time follow-ups; and (perhaps) a favorable immunological and intracellular molecular situation. These first successes have encouraged many researchers to believe that less favorable starting points in other cancer types can be overcome, leading to clinical efficacy.

F. Hematological and Lymphatic Malignancies

These malignancies are the domain of chemotherapy. Due to the development of advanced protocols, the initial rate of remission and the number of long-term survivors have dramatically increased. In all stages, the primary therapeutic intention is curative. The usual treatment regime consists of several cycles of intensive or high-dose chemotherapy up to a full eradication of the patient's bone marrow followed by autologous or allogeneic bone marrow transplantation. Due to the good susceptibility of leukemic and lymphatic cells for chemotherapy, conventional treatment usually leaves only minimal residual disease. This residual tumor load can, however, lead to relapses in up to 80% of patients with leukemia (342) and has usually a bad prognosis. Gene therapy could try to prevent this situation.

Several immunological approaches have been tried. Preclinical, in vivo gene transfer of TNF-alpha was used for T-cell lymphoma (343) and myeloma (344), B7 for several lymphomas (345,346), and GM-CSF for T-cell leukemia (347). Transfer of IL-2 into lymphomas is already in a clinical phase I study (348).

Human primary hematological malignant cells are unfortunately highly resistant to transduction by most available vectors. Only recently, modifications of vectors and ex vivo transduction protocols have allowed increased gene transfer—at least into cultured allogeneic vaccine lines with adenovirus, HSV, liposomes, and retroviral vectors. Another problem is the considerable phenotypic heterogeneity of the cancer, which can cause an incomplete representation of potential antigens of the whole malignant cell population in the population selected for gene transfer and vaccination.

Despite these problems, a considerable number of cancer gene therapy trials using ex vivo cytokine-transduced vaccines have been conducted. Among several cytokines, IL-4 and GM-CSF protected best against tumor challenge, and GM-CSF-transduced cells could also inhibit further progression of preestablished lymphatic tumors (349) in a mouse model. Most clinical trials used IL-2, but in others IL-4, 7, 12, IFN-γ, and GM-CSF vaccines were applied.

Vaccinations with the DNA of tumor antigens have also been carried out. Gene therapy provides an advantage here over the use of the respective protein when this is difficult to obtain in the required amount or in the correctly glycosylated form. Encouraging results were obtained in a clinical phase I study of the vaccination of patients with chronic lymphatic leukemia (CLL), with CLL cells adenovirally transduced to overexpress the CD 40 ligand (CD 154); a phase II trial has been initiated (350).

Ex vivo gene transfer into effector cells has also been examined clinically. In a phase I clinical trial, autologous, cytokine-induced killer (CIK) cells ex vivo transduced with IL-2 were infused in patients with metastatic disease. One out of 10 patients had a complete response to the lymphoma (351).

The generation of chimeric T-cell receptors (TCRs) has been another strategy to generate antitumor immunity for B cell lymphoma. T cells are directed against target cells by grafting an antibody V region of desired specificity onto the TCR and the constant regions. The specificity of this approach in vitro has been shown (352).

The transfer of donor leukocytes (353) or EBV-specific cytotoxic T lymphocytes (354) has been a successful approach for the treatment of B cell lymphoma. A potential problem of this strategy, however, could be graft vs. host disease (GvHD). An ingenious way to circumvent this problem is the transduction of the donor T cells with a suicide gene such as HSV-tk to be able to kill the donor T cells by GCV if signs of GvHD appear (355,356). In a clinical trial, GvHD resolved in 2 of 3 patients upon application of GCV. A recent review of the pros and cons of this strategy can be found at (357).

One sustained response in a clinical trial was observed upon the application of TILs engineered to secrete TNF (358).

For therapy of multiple myeloma, direct intratumoral gene transfer of the TNF gene was performed preclinically and a ''substantial gene transfer in nearly 50% of tumors was seen'' (359). Anecdotally, 1 woman received an injection of an IL-2 adenovirus into an occipital myeloma lesion, and gene transfer could be demonstrated although no tumor regression was observed (360). In fact, myeloma cells seem to be among the easiest transducable hematological cells, at least for adenoviruses (361) and AAV (362).

Lymphomas and leukemias have also been targeted with oligonucleotides against myc (263,363,364), bcl-2 (365), myb (366), bcr-abl (363,364), and bcr-abl ribozymes (367).Transfer efficacy of antisense oligonucleotides in vitro and in vivo is highly controversial, but the first clinical trial produced remarkable efficacy with nearly half of the patients showing stale disease and 1 patient being in remission for at least 3 years (Lattime 136, 137).

Therapy-associated side effects like infections and hemorrhages account for 70% of the deaths of adult patients with acute leukemia (368). The transfer of MDR genes into hematopoietic stem cells for chemoprotection holds promise, and clinical studies of the transduction of the MDR gene in patients with relapsed and resistant lymphomas are already under way. This approach, however, runs the risk that tumor cells are transduced, making them resistant to chemotherapy. Only the

future will show whether this problem can be neglected or circumvented (e.g., by targeted vectors).

In summary, it comes as no surprise that hematological malignancies as a systemic disease are the domain of immunological approaches, which have demonstrated clinical success in several cases.

G. Hepatocellular Carcinoma

Although hepatocellular carcinoma (HCC) is of moderate epidemiological relevance in the Western world, it is the most common cancer in large areas in Asia. HCC often remains localized to the liver, but only a minority of patients is amenable to local therapy such as surgery, liver transplantation, or cryo-ablation. Standard chemotherapy is largely ineffective. Therefore, HCC is a suitable target for intratumoral application of therapeutic genes. As demonstrated, adenovirus vectors can easily infect and express different types of genes in tumor cells of HCC (28,95). In addition, the alpha-fetoprotein (AFP) promoter is extremely HCO-specific (369) for achieving tumor-specific gene expression. Using adenovirus mediated gene transfer, different experimental approaches were studied demonstrating significant tumor volume reductions by IL-12 (370), HSV-tk/GCV (371,372), and oncolytic adenovirus (373); combinatorial expression of p53 and p16 (28); or p21 and GM-CSF using an EGF-receptor targeted adenovirus (374).

A pilot clinical study to assess the therapeutic potential of percutaneous intratumoral injection of wild-type p53 (wt-p53) in patients with primary hepatocellular carcinoma was initiated in 1996. Nine patients with primary hepatocellular carcinoma and 6 patients with colorectal liver metastases received percutaneous injections of a wild-type p53 DNA-liposome complex. In contrast to nonresponders with colorectal metastases, 4 of 9 patients with HCC showed a reduction of tumor volume and a significant decrease in serum AFP levels (375, 288). Phase I and II trials were conducted in which injection of B7-1 costimulatory molecules resulted in 12 of 33 patients having a significant drop in AFP levels. Three patients had a greater than 50% decrease in tumor size, allowing later surgery of initially inoperable tumor (376). A phase I trial was recently published that compared intratumoral injection of ethanol with that of conditionally oncolytic, replication-competent, E1B55K-deleted adenovirus. In the gene therapy group, 1 patient showed a partial response and 4 patients had progressive disease. In the ethanol-treated group, 2 patients had stable disease and 3 patients showed disease progression (377).

H. Lung and Pleural Cancer

In the Western world, lung cancer has become the most frequent tumor for males (368). The main reason for this is smoking; more than 90% of these patients are or were smokers. The prognosis of this cancer is bad. Due to regional or systemic metastases, tumors can be resected in only 25% of the patients. Only one-fourth of these patients will survive 5 years; only 6% of the patients are curable. Because of the tendency for early metastasis, gene therapy approaches aiming at a systemic response such as immuno-gene therapy are needed. A potent evocation of an immune response has only recently been reported in animal experiments (378). Mesothelioma is a rare type of cancer originating from the pleura.

Because lung cancer metastasizes early, this tumor is a very difficult target for gene therapy. However, the high epidemiological relevance of lung cancer creates an urgent need to develop alternatives to the standard therapeutic approaches. This is especially true for non small cell lung cancer (NSCLC). Because NSCLC is in most of the cases highly resistant to any kind of chemotherapy, and well-documented studies about genetic defects in NSCLC exist, this tumor was from the beginning an attractive target for the use of tumor suppressor genes, e.g., p53. Impressive results were obtained in preclinical studies: significant tumor growth inhibition in subcutaneous and orthotopic animal models (379,380). Therefore, one of the first cancer gene therapy trials was initiated to treat NSCLC by gene transfer of p53. A retroviral vector containing the wild-type p53 gene under control of a beta-actin promoter was used. Nine patients for whom conventional treatments had failed received direct injections into the tumor. Despite a low efficacy of gene transfer, and lacking evidence for an involvement of T-cell mediated immunity, partial tumor regression was noted in 3 patients, and tumor growth was stabilized in 3 other patients (381). To improve the in vivo gene transfer efficacy, adenoviral vectors were used for p53 gene transfer in 3 additional studies (382–384), and in 3 more studies p53 adenovirus was combined with chemotherapy (385–387). In several patients in these trials, transient local control was observed. This was clearly attributed to the gene therapy treatment.

Lung cancer has also been a model disease for antioncogene therapy. The K-ras oncogene is frequently overexpressed in lung cancer. Intratracheal transfer of retroviruses carrying an antisense K-ras construct markedly reduced tumor size and the number of lung tumors in nude mice (388). Another candidate for antisense therapy is the ErbB2 transmembrane protein kinase receptor whose aberrant expression has been shown to contribute to malignant transformation and progression. The promoter of the ErbB2 gene could be used for expression of toxic transgenes. Promoters that are activated by ionizing radiation (389) have also been used for this purpose. Researchers hope that sufficient specificity for tumor deposits will be achieved in the future, enabling even metastases to be efficiently transduced without toxicity for the surrounding normal tissue. Under these circumstances, the combination of gene therapy with conventional therapy could be a particularly attractive strategy as discussed above for p53 and cisplatin.

To date, there has been no published trial using suicide-gene therapy in patients with lung cancer. However, there are 2 reports of the treatment of patients with pleural melanoma (390,391). In both trials, HSV-tk adenovirus was injected intratumorally and only minimal side effects were observed. A marked difference between the trials was that in 1 study a high dose of glucocorticosteroids was applied to counteract vector-related immune responses (64). In this study, partial

tumor regressions were observed in some patients; 2 of them even remained tumor-free 3 years after treatment.

Several phase I and II immuno-gene therapeutic clinical trials for the treatment of lung cancer have been conducted using the adenovirally GM-CSF-transduced, allogenic, NSCLC cell line GVAX (392). An interim analysis of 30 patients from a multicenter phase I and II trial demonstrated a major response rate in 18% of the patients, 10 of whom showed complete tumor remissions (393).

Partial regression of a lung mass was observed in 1 of 10 patients treated with IL-2 transfected TILs (394). Another immuno-gene therapeutic approach with promising preclinical results is the application of adenovirally IL-7-transduced DCs for the treatment of lung cancer (395). A clinical trial of direct intratumoral injections of vaccinia virus harboring IL-2 for the treatment of pleural mesothelioma led to diverse immunological responses, although no significant tumor regressions were seen (396). In summary, some clinical efficacy with cancer gene therapy has been demonstrated. However, this is clearly neither satisfactory nor sufficient for this devastating disease.

I. Melanoma

Malignant melanoma is a tumor of average incidence worldwide but with extremely high incidence in certain areas such as Australia. Worldwide, an annual increase in incidence of 6–7% was reported (397). Melanoma is a tumor with a very high resistance to treatment. Treatment schedules, including radiation, chemotherapy, and combinations of both, have no significant impact on the overall survival of the patients (398,399). Although the primary tumor can usually easily be excised, distant metastases cause the death of nearly all patients. Early and fatal metastazation is 1 reason why this disease is an important target for approaches like immunotherapeutic therapies from which a systemic antitumor efficacy can be expected. Fortunately, melanoma displays a naturally high immunogenicity, which facilitates the recognition of the tumor by the immune system.

Basically, all principles of immuno-modulation described at the beginning of this chapter have been applied to melanoma. Trials in melanoma alone account for 54% of all open immuno-gene therapy trials in the U.S. At the time of writing, the number of clinical immuno-gene therapy studies for melanoma worldwide exceeds 50. The earliest trials were based on the transfer of TNF-transduced TILs (6). Phase I trials are now closed, but due to minor success no further trials entirely focusing on TILs have been initiated. However, in a later trial, TILs obtained from a patient's tumor nodules were injected with the HLA-B7 molecule, expanded and reinfused, and direct immunological effects were demonstrated (400). The use of chimeric T-cell receptors with cytokine or melanoma antigen domains is an interesting approach in the area of adoptive gene therapy with genetically modified, immune cells. In this approach, CD8 + T-cells are redirected to the tumor and create a favorable cytokine-rich environment (401).

Recently, much has been expected from adoptive transfer of dendritic cells (DCs), which are probably the most potent antigen-presenting cells in the body. They are also easy to transfect with several vectors. DCs have been transduced ex vivo with tumor antigens and costimulatory molecules. MART-1, antigen-transfected, dendritic cells in particular generated MART-1-specific immunity and arrested the growth of established tumors (402,403). Recently, it has been clearly shown that $CD8^+$ T-cells receive CD4 help directly through the receptor CD40 and that this interaction is fundamental for $CD8^+$ T-cell memory generation (404). Intratumoral injections of DCs that had been transduced in vitro with CD40-ligand led to regression of injected, as well as distant, B16 melanoma nodules (405).

The majority of preclinical and clinical data for melanoma immuno-gene therapy has been generated in vaccination trials. Vaccination is performed traditionally with autologous tumor cells, but allogeneic vaccines consisting of autologous fibroblast or standardized gene-transduced cell lines are used as well (reducing cost and time). Many human trials using either cytokines (e.g., IL-2,-4,-6,-7,-12, IFN-α, and IFN-γ) or costimulatory molecules have been completed or are still ongoing. The best results in clinical vaccination trials have probably been obtained with granulocyte-macrophage, colony-stimulating factor (GM-CSF); this had already shown the greatest degree of systemic immunity among 10 different cytokines in preclinical models (406). In a melanoma vaccination trial with GM-CSF-transduced, autologous, melanoma cells, extensive tumor destruction and pronounced immunological responses were observed in 11 of 16 patients (407). GM-CSF was also transferred intratumorally using vaccinia virus (485) or transfected into tumor cells to prime tumor-derived lymph node cells in a trial of adoptive gene transfer (486). Excellent responses, including complete remissions, have been obtained in both types of clinical studies.

More than 50 patients in 5 different studies have received intratumoral injections of HLA-B7 DNA using liposomes (348,408). DNA or protein was detected in the great majority of the patients. Toxicity not attributable to the mechanical irritations was the exception, and local or even general responses were seen in one-third of the cases.

Published results of a trial involving multiple intratumoral injections into melanoma metastases of a retroviral vector carrying the IFN gene showed either stable disease or partial or complete response of the injected lesions (409).

Besides the immunological approaches illustrated above, which clearly dominate gene therapy of melanoma, preclinical suicide-gene (Table 1) and tumor-suppressor gene (p53) (Table 1) therapies have also been performed. Combinatorial approaches (e.g., cotransfer of suicide genes and cytokines) have resulted in additive, tumor growth inhibition (344,410). A clinical phase I/II trial (411) for suicide therapy of metastatic melanoma with retroviral HSV-tk vectors resulted in significant tumor necrosis in 3 of 8 patients despite a low transfection efficacy of less than 1%.

Intratumoral injections of a p53 adenoviral vector for the treatment of patients with breast and melanoma metastases were associated with minimal toxicity and the detection of p53 mRNA in biopsies of injected tumors (412).

In the preclinical stage, treatment of melanoma cells with oncogene Ha-ras (413) or c-myc (414)-specific antisense oligonucleotides led to good antitumor efficacy in SCID mice. The application of dominant, negative Stat3 cancer-related gene led to tumor regressions due to massive apoptosis (414). In 1 clinical trial for the treatment of malignant melanoma with dacarbacin and antisense-bcl-2, one complete remission has been observed (487).

In summary, the preclinical and in particular the clinical results obtained with immuno-gene therapy of melanoma are among the most encouraging findings in the field of cancer gene therapy.

J. Osteosarcoma

Osteosarcoma primarily afflicts young people within the first decades of life and accounts for 5% of all childhood malignancies (415). The overall 2-year metastasis-free survival rate approaches 66% (416,417). Metastases, mainly to the lung, are the predominant cause for mortality. Therefore, immunological approaches such as the in vivo transfer of the B7 gene (418) may be particularly fruitful. However, the osteocalcin promoter constitutes an available tissue-specific promoter, which potentially allows systemic or regional treatment with cytotoxic genes. It has already been proven to be efficient in vivo in an adenoviral context (419). The osteocalcin promoter expressing the adenoviral E1A gene to allow for tumor-specific replication will also be used for intravenous application in a clinical trial for the treatment of metastasized osteosarcoma (420). Other successful preclinical approaches include aerosolic adenovirus-IL-12 (421), PEI-IL-12 (422), or PEI-p53 complexes for the treatment of lung metastases; adenovirus-IL-3-β gene transfer for isolated limb perfusion (423); and intratumoral injection of retrovirus VPCs-HSVtk (424).

K. Ovarian Cancer

Ovarian cancer is the leading cause of death from gynecological malignancies in women (419). Due to improvements in surgery, radiation, and chemotherapy techniques, the 5-year survival rate has improved over the last 20 years. However, over two-thirds of the patients have advanced stage disease at presentation; despite transient responses, the long-term survival of these patients rarely exceeds 15% to 30%. Although even patients with advanced stages often have their disease confined to their abdomen for extended periods of time, intraperitoneal chemotherapies have only moderate success. Basically, this is because they do not provide the reduced toxicity profiles initially hoped for. Therefore, ovarian cancer has become one of the model diseases for gene therapeutic approaches with intracavital vector applications.

Several clinical studies have been initiated with HSV-tk as transgene in which vectors or modified cells were applied intraperitoneally. Transfer of adeno-HSVtk at doses up to 1×10^{11} pfus led to stable disease in 5 of 14 patients and transient fever in 4 patients. Transient hematological grade 3 and 4 toxicities were observed if an adeno-HSV-tk was combined with topotecan chemotherapy, but since toxicity was not related to vector dose, these side effects are more likely related to the chemotherapy (425). In a vaccine/suicide trial with patients receiving irradiated PAI-1 ovarian cancer cells ex vivo transfected with HSVtk, a high incidence of fever and abdominal pain was seen in several patients, along with resolution of ascites or decreased CA-125 levels (426). Results of a trial using retroviral VPCs (427) have not yet been published.

Phase I trials have also been initiated for adenoviral delivery of an anti-erbB-2, single-chain antibody gene (428,429). This ''intrabody'' approach could become a potent alternative to antisense strategies. In a clinical trial, adeno-anti-erbB-2 was injected intraperitoneally. Despite the relatively low dose of up to 1×10^{11} pfus, 5 of 13 evaluable patients had stable disease (425).

The results of 2 trials using p53-adenovirus have been published. In 1 trial, 2 of 11 patients had a partial response, 4 patients had stable disease, and no major toxicity was reported despite multiple injections of up to 3×10^{12} viral particles (430). In the second phase I/II trial enrolling 36 patients, adeno-p53 was administered either alone or combined with chemotherapy at single or multiple doses. A comparison with historical controls led the authors to suggest 1 of 2 explanations: either the combination of chemotherapy and gene therapy had a synergistic effect; or gene therapy changes the nature of the cancer, contributing to a chronicity of the disease that favorably impacts survival (431,432).

A trial with replication-competent adenovirus showed significant toxicity but no efficacy (433).

Finally, intraperitoneal injection of a retrovirus harboring a BRCA-1 splice variant showed partial responses in 3 of 12 patients in a phase I study. However, the lack of any response led to an early termination of a subsequent phase II study (434,435). The authors discuss differences in tumor burden and immune system status of the patients as possible reasons for the differing response to BRCA1 gene therapy.

Further interesting approaches with significant growth retardations of ovarian carcinoma have been demonstrated in preclinical experiments. These include liposomal transfer of E1A (436) or mutant SV 40T antigen (437). Preliminary data from a clinical trial for intracavital application revealed (279) HER-2/neu downregulation, increased apoptosis, and reduced proliferation. The most common treatment-related toxicities were fever, nausea, vomiting, and/or discomfort at the injection sites.

In summary, intracavital gene therapy of ovarian cancer showed clinical antitumor effects with immunological and nonimmunological approaches.

L. Pancreatic Cancer

This disease has a low incidence but a very bad prognosis, mainly because the primary tumors are not resectable at the

time of discovery. It accounts for only 2% of all newly diagnosed cancers in the United States but for 5% of all cancer deaths (438). Moreover, the tumors are highly chemoresistant. Partial remissions of up to 15% of the cases have only recently been seen with the new drug gemcitabine (368).

More than 80% of pancreatic tumors contain a mutation of the ras oncogene in position 12 (439). Since this mutation (as well as others) is recognized by the immune system, ras-peptide-based immunotherapy has been successful in mice. Clinical studies have been performed and an association between prolonged survival and an immune response against the vaccine was seen (440–442). Recently, antigen-presenting cells, genetically modified to express tumor antigen, were shown to induce antigen-specific cytotoxic T cell responses in vitro and in vivo (443). H-ras antisense oligos have also been used in a phase II clinical trial, and clinical stabilization was seen in 2 out of 16 patients (444).

Because the local disease is often life-limiting in pancreatic cancer, the disease is attractive for cytotoxic gene therapy approaches (445–447). A combination of HSV-oncolysis and HSV-tk/GCV cytotoxicity increased the percentage of mice with peritoneal pancreatic tumors, obtaining significantly prolonged lifespan to 70% as compared to 40% with oncolysis alone (448). A very interesting trial used ex vivo suicide, gene-transduced, autologous tumor cells for localized activation of chemotherapy. Genetically modified allogeneic cells, which expressed a cytochrome P450 enzyme, were encapsulated in cellulose sulphate and delivered by supraselective angiography to the tumor vasculature. These cells locally activated systemically administered ifosfamide. The tumors of 4 of 14 patients regressed after treatment, and those of the other 10 individuals who completed the study remained stable. Median survival was doubled in the treatment group by comparison with historic controls, and 1-year survival rate was 3 times better (449).

Effective preclinical gene therapy for pancreatic cancer was also seen by combining oncolytic, conditional, replication-competent adenoviruses and IL-2 or IL-12 adenoviruses (450). Replication-deficient IL-2 adenoviruses were used in a phase I/II trial for digestive cancer, and a single intraoperative intratumoral injection did not cause any adverse effects (451).

In a phase I study, allogenic pancreas cells engineered to secrete GM-CSF were intradermally applied, and 3 patients who had received higher doses of cells seemed to have increased survival times (452).

M. Prostate Carcinoma

Prostate cancer (PCA) is the most frequently diagnosed cancer in men in the U.S. and the second leading cause of death from malignancy (453,454). Locally restricted tumors can be treated by surgical resection or radiotherapy. Androgen-ablative therapy often induces dramatic responses, but virtually all patients progress to an androgen-refractory state with a median survival of 12–18 months. The prostate is a unique accessory organ and expresses several hundred unique gene products as potential targets for gene therapy. Prostate cancer can therefore serve as a model disease for tumor-specific gene therapy. The efficacy and specificity of the PSA promoter has been proven in animal experiments (455). This promoter has also been used to confer prostate-specific replication of conditionally replication-competent adenoviruses (50). A clinical trial using this vector for injection into locally recurrent prostate carcionoma provided relative safety with no grade 4 toxicity and a more than 50% reduction in PSA levels in all patients treated with the highest dose (456).

More than 20 immuno-gene therapeutic trials for prostate cancer have been initiated, and some results have been published. A GM-CSF-secreting, autologous PCA vaccine increased antibody titers against prostate tumor antigens (457); direct intratumoral injection of liposomal IL-2 plasmid led to decreases in serum PSA levels in 80% of patients (458); and vaccinia virus MUC-1-IL-2 also decreased PSA levels (458).

As an example of gene-replacement therapy, a trial with direct injection of retrovirus-BRCA-1 should be mentioned: while it did not reveal any antitumor efficacy, it did at least demonstrate the safety of the approach (459). Clear objective responses were observed when adenovirus-HSV-tk was injected into the tumorous prostate. At the highest treatment dose, PSA levels declined by 50%. However, reversible grade 4 thrombocytopenia and grade 3 hepatotoxicity occurred (460).

Successful preclinical experiments have been reported for the transfer of the antiIGF-1 receptor (461), p21 (462), p53 (462,463), p16, and antisense myc (464). Three gene therapeutic clinical trials have already been initiated with antisense myc.

The future use of prostate-specific regulatory elements and target genes holds promise in the treatment of disseminated disease, the major cause of death in prostate cancer patients. Treatment will eventually be possible if systemic application of the vectors harboring cytotoxic genes can be done without major toxicity.

V. CONCLUSIONS

We have now seen nearly 15 years of preclinical gene transfer and more than a decade of clinical gene therapy. A wide variety of vectors and transgenes has been successfully tested in animals. Several types of vectors and many different therapeutic principles have been evaluated clinically, producing 403 studies of cancer gene therapy (Tables 2–4).

The general outcome of these clinical studies indicates that gene therapeutic vectors are usually tolerated with mild-to-medium acute toxicity. The toxicity has been reversible in all cases. No cases of long-term toxicity have been reported. In the majority of clinical trials, at least some efficacy was seen. In some trials, a clear benefit for the patient was observed in comparison with either historical controls or (in some exceptional cases) with internal controls. Apparently, in all cases where the best results in clinical trials have been achieved, the cancer target was naturally most well suited to current cancer gene therapeutics. Examples of this are the good accessibility of superficial bladder cancer for intravesically in-

(*text continues on p. 566*)

Table 2 Cancer gene therapy, in vivo gene transfer, preclinical studies, 1990–1998

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
Anti-Oncogenes							
	c-fos	Retro	i.p.	5×10^5	80% (weight)(1)	nude(5), breast cancer (MCF-7), s.c., 1–4 mm^3(6)	[5]
	anti ErbB-2 antibody	Ad	i.p.	500 pfu/cell	12-fold decreased risk of death	SCID, ovarian cancer (SKOV-3), i.p.	[28]
	anti-K-RAS	Lip		100 μg(3), 3×(7)	83% (cures)(4)	nude, pancreatic cancer (AsPC-1), i.p.	[4]
	anti-Cyclin G1	Retro	i.tu.	1×10^7, 10×	79% (vol)(1)	nude, osteosarcoma (MNNG/HOS), s.c., 50–60 mm^3	[20]
	bcl-x_4	Ad	i.tu.	7×10^7	50% (vol)	nude, breast cancer (MCF-7), s.c.	[30]
Tumor Suppressor Genes							
	p53	Ad	i.tu.	2.2×10^8, 10×	231: 86% (vol) 468: 74% (vol) 435: n.s. (vol)	nude, breast cancer (MDA-MB-231, −468, −435), s.c. or ortho(8)	[64]
	p53	Lip	i.v.	1×16 μg, 1×12 μg	MDA-435: 75% (vol) MCF-7: 40% (vol)	nude, breast cancer (MDA-MB-435: 17.6 mm^3, MCF-7: 12.8 mm^3), ortho	[99]
	p53	Lip	i.v.	35 μg, 6×	60% (vol) 97% (metastasis)	nude, breast cancer (MDA-MB-435) ortho	[54]
	p53	Ad	i.tu.		45% (cures)	SCID, ovarian cancer (SK-OV-3), s.c., 5–6 mm	[38]
	p53	Ad	i.tu.	$1–2 \times 10^8$, 6–8×	> 65% (cures)	nude, prostate cancer (C4–2), s.c.	[49]
	p53, p21	Ad	i.tu.	5×10^9	p21: 68%, p53: 21% (vol)	129/SV mice, prostate cancer (148–1PA), s.c., 24–40 mm^3	[32]
	p53	Ad	i.tu.	5×10^9, 6× CDDP, i.p.	83% (vol) 91% (vol)	nude, lung cancer (H1299), s.c., 250 mm^3	[63]
	p53	Ad	i.tu.	2×10^9, 8×	97% (vol)	nude, lung cancer (SCLC, NIH-H69), s.c., 40 mm^3	[95]
	p53	Ad	i.tra.	5×10^7, 2×	73% (vol)	lung cancer (NSCLC, H226Br)	[102]
	p53	Ad, cisplatin	i.tu.	2×10^7	74% (vol)	nude, lung cancer (NSCLC, H358), s.c., 5–6 mm	[37]
	p53	Retro	i.tra.	100 μl, 3×	64%–100% (vol)	nude, lung cancer (NSCLC, H226Br)	[36]
	p53	Ad	i.tu.	10^8	100% (cures) MDA 886: 33% (cures)	nude, head and neck cancer (Tu-138, Tu-177, MDA 686-LN, MDA 886), s.c., microscopic	[26]
	p53	Ad	i.tu.	10^8	98% (vol)	nude, head and neck cancer (Tu-138, Tu-177), s.c., 6 mm	[58]

(*Continued*)

Table 2 Continued

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
	p53	Ad	i.tu.	1×10^7	tumor growth suppression	nude, head and neck cancer, SCCHN (MDA686LN), s.c.	[57]
	p53	Ad	i.tu.	10^4	40% (vol)	Wistar rat, glioma (9L), ortho	[6]
	p53	Ad	i.tu.	10^9, 5×	68% (vol)	nude, colon cancer (DLD-1), s.c.	[42]
	p53	Ad	i.tu.	$3{,}3 \times 10^9$, 3×	SW620: 62% (vol) KM12L4: 69% (vol)	nude, colorectal cancer (SW620, KM12L4), s.c., 200 mm^3	[81]
	p53	Ad	i.tu.	2×10^9	B16: 38% (vol) SK: 24% (vol)	nude, melanoma (B16-G3.26, s.c., 1200 mm^3, SK-MEL-24, s.c., 300 mm^3)	[24]
	p53	Ad	i.a. (hepatic artery),	4×10^9, 4×	> 64% (tu nodules)	Buffalo rat, HCC (McA-RH7777), ortho	[3]
	E1A	Lip	i.p.	15 μg DNA, weekly	400% (surv)$^{(2)}$	nude, ovarian cancer (SKOV-3), i.p.	[101]
	E1A	Ad	i.v.	10^8, 6×	71% (vol)	nude, lung cancer (NCI H820), ortho	[19]
	mutant SV40 T Ag	Lip	i.p.	15 μg DNA 30×	incr survival (40% > 1 year vs. 0% > 3 month (control)	nude, ovarian cancer (SKOV-3), i.p.	[97]
	p21	Ad	i.tu.	5×10^9	p21: 68% (vol), p53: 21% (vol)	129/Sv, prostate cancer (148-IPA), s.c., 25–40 mm^3	[31]
	p21	Retro-VPCs	i.tu.	1×10^6, 3×	58% (vol)	nude, squamous carcinoma (HN8), s.c., 3 mm^3	[16]
	Rb	Ad	i.tu.	5×10^7 particles	125% (surv)	$Rb^{+/-}$ mice, pituitary cancer, ortho	[77]
	truncated Rb	Ad	i.tu.	5×10^8, pfu, 6×	95% (vol)	nude, bladder cancer (5637) s.c.	[98]
	BRCA-1	Retro	i.p.	titer: 10^7/ml	300% (surv)	nude, breast cancer (MCF-7), i.p., 3–5 mm	[46]
	bcl-x_s	Ad	i.tu.	7×10^7 pfu, 4×	50% (vol)	nude, breast cancer (MCF-7), s.c.	[30]
Prodrug-activating Genes	HSV-tk	AAV	i.tu.	$9{,}6 \times 10^9$, 3×	> 80% (cures)	nude, glioma (U251-SP) ortho, 2 mm	[61]
	HSV-tk	Ad	i.tu.	10^8 pfu	83% (vol)	C57BL6, pancreatic cancer (PANC02), to liver, 4,5 mm	[10]
	HSV-tk	Ad	i.port.	2×10^{10}	63% (cures)	Wistar/Ico rats, HCC (DENA induced), multiple 1–7 mm	[73]
	HSV-tk	Ad	i.tu.		84% (vol)	SCID, colon cancer (LS 174), s.c.	[13]
	HSV-tk	Ad	i.tu.	3×10^8	>75% (vol)	C3H/He, bladder cancer (MBT-2), s.c., 40 mm^3	[84]
	HSV-tk	Ad	i.tu.	5×10^8	66% (weight), 112% (surv)	C57/BL6, prostate cancer (RM-1), ortho	[41]
	HSV-tk	Ad	i.tu.	5×10^8	84% (vol), 150% (surv)	C57/BL6, prostate cancer (RM-1), s.c., 50 mm^3	[31]

HSV-tk	Ad	i.p.	2×10^9, 3×	> 300% (surv)	nude, ovarian cancer (Ov-ca-2774), i.p.	[90]
HSV-tk	Ad	i.p.	10^9, 2×	91% (weight)	nude, breast cancer (MCF-7), i.p.	[21]
HSV-tk	Ad	i.tu.	10^9, 2×	HuH7: (cures), SK-Hep-1: (red vol)	nude, HCC (Huh7: 83, 2 mm^3 SK-Hep-1: 10,9 mm^3), s.c.	[48]
HSV-tk	Ad	i.tu.	10^{10}	40%–50% (vol)	nude, melanoma (B16), s.c., 6–8 mm	[11]
HSV-tk	Ad	i.tu.	$1{,}2 \times 10^9$	100% (cures)	Fisher rats, glioma (9L), ortho, 1,7 mm^2	[70]
HSV-tk	Ad	i.tu.	3×10^8	99,8% (vol)	nude, glioma (C_6), ortho, 4 mm	[23]
HSV-tk	Ad	i.thek.	2×10^9	126% (symptom free latency)	Fisher rat, glioma (9L), ortho	[93]
HSV-tk	Ad	i.p.	$6{,}5 \times 10^8$	340% (surv) 28% (weight)	nude, breast cancer, (MDA-MB-435A), i.p.	[100]
HSV-tk	HSV	i.thec.	10^8	90% (cures)	Fisher rats, glioma (9L), ortho	[50]
HSV-tk	HSV-tk-pos. tumor cells	i.tu.	1×10^5	retardation of tumor growth	Fisher rat, glioma (9L)	[62]
HSV-tk	Lip	i.tu.	3 μg, 14×	69% (vol)	nude, colon cancer (Colo201), s.c.	[86]
HSV-tk	Lip	i.tu.	3 μg DNA, 10×	Colo 320DMO: 57%, A-431: 75%, Nakajima, KF: not sig (vol)	nude, colon cancer (Colo 320 DM), vulva (A 431), ovarian cancer (Nakajima, KF), s.c., 40–60 mm^3	[82]
HSV-tk	Plasmid	i.tu.		40–50% (weight)	C57B1/6, melanoma (B16F1) s.c.	[80]
HSV-tk	Retro	i.tu.	10^8	29% (cures)	Fisher rat, glioma (9L), ortho	[51]
HSV-tk	Retro-VPC	i.tu.	3×10^6	79% (cures)	Fisher rat, glioma (9L), ortho, micro	[27]
HSV-tk	Retro-VPC	i.tu.	5×10^5	140% (surv)	Fisher rat, glioma (9L), ortho	[74]
HSV-tk	Retro-VPC	i.tu.	2×10^7	95% (vol)	nude, head and neck cancer (UMSCC 29), s.c., 5 mm	[96]
HSV-tk	Ad,	i.tu.	Ad:	Ad>Retro-VPC (surv)	Fisher rat, glioma (9L), ortho	[94]
	Retro-VPC		5×10^3 pfu, Retro VPC: 5×10^6			1996 HGT
CD	Ad	i.v.	10^9	97% (vol)	BALB/c, colon cancer (CT26) to liver, ortho, microscopic	[91]
CD	Ad	i.p.	1×10^9	64%–85% (weight)	nude, i.p. gastric cancer (MKN45)	[53]
CD	Ad	i.tu.	10^9	70% (vol)	C57BL/6, pancreatic cancer (PAN02), s.c.	[33]
CD	Ad	i.tu.	1×10^9	81% (vol)	nude, breast cancer (MDA-MB-231), s.c.	[56]
CD	Ad	i.tu.	10^9	70%–85% (vol)	nude, HCC (PLC/PRF/5), s.c., >100 mm^3	[47]
CD	Ad	peri.tu.		growth suppression	nude, colon cancer (HT29) to liver, ortho	[68]
CD	Lip	i.tu.	10 μg or 50 μg, 10×	incr (surv)	C57/BL6, melanoma (B16(F10)), s.c.	[85]

(*Continued*)

Table 2 Continued

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
Immunotherapeutic Genes	MHC II	tumor cells			60% (cures) (2–5 mm) 40% (cures) (4–7 mm)	A/J, sarcoma (Sal), s.c., 2–5 mm, 4–7 mm	[8]
	MHCI H-2Ks	Lip	i.tu.	1 μg, several times	9/12 tu. growth retardation	BALB/c, colon cancer (CT-26), s.c.	[72]
	IL-2	tumor cells		2×10^6, 5×	70% (vol)	C57BL/6, pancreatic cancer (Panc 02), s.c.	[25]
	IL-2	tumor cells	s.c.	2×10^6	64% (vol)	nude, melanoma (DM92), s.c.	[2]
	IL-2	tumor cells	s.c., i.p., i.pleu.,	5×10^6, 3×	no effect (s.c.) 80% (cures, i.pleu.) 100% (cures, i.p.)	C57BL/6, lung cancer, (Lewis lung carcinoma), i.p., i.pleu, s.c.	[44]
	IL-2	fibroblasts	s.c., i.cr.		incr surv., i.cr. >i.s.	C57BL/6, glioma (GL261), ortho	[39]
	IL-2	Ad	i.tu.	2×10^9, 3×	65% (diameter)	C3H, fibrosarcoma (FSA), breast cancer (MCA-K), s.c., 3–5 mm	[89]
	IL-2	Ad	i.tu.	2×10^9, 3×	95,2% tu growth delay	SCID, HCC (HepG2, Hep3B), 4–6 mm	[14]
	IL-2	fibroblasts	s.c.	2×10^6, 4×	44% (cures)	nude, colon cancer, (CT-26), s.c.	[34]
	IL-4+systemic IL-2	fibroblasts	s.c.	10^6	100% (tumor growth delay)	C57BL/6, fibrosarcoma (MCA105)	[71]
	IL-4	tumor cells	s.c.	4×10^6	180% (surv)	C57BL/6, melanoma (B16), s.c.	
	IL-4		s.c.	1×10^6, weekly 3×	70% (cures)	BALB/c, renal cancer (Renca), s.c.	[29] [40]
	IL-4	Lip	i.tu.	10 μg	89% (vol)	C57BL/6, melanoma (B16(F10)), s.c.	
	IL-12	tumor cells	s.c.	5×10^6	100% (cures)	BALB/c, fibrosarcoma (CMS5a), s.c.	[60]
	IL-12	tumor cells	s.c.		d1: 30% (cures), d7: 10% (cures)	BALB/c breast cancer (TSA), microscopic (d1), 2, 4 mm (d 7)	[79] [18]
	IL-12	Ad	i.tu.	5×10^8	77% (vol)	BALB/c, colon cancer (MCA-26), to liver, ortho, 16–25 mm^2	[17]
	IL-12	Gene gun	i.d.	5 μg, 2×	94% (diameter),	BALB/c, sarcoma (Meth A), i.d.	
	IL-12	Gene gun	i.d.	5 μg, 2–4×	P815: 28% (vol), B16: 47% (vol) (cures): Renca, L5178Y: 87,5%, MethA: 57%, SA-1: 37,5%	BALB/c: renal cancer (Renca), sarcoma (Meth A), i.d. Sarcoma (SA-1), DBA 2 mice: lymphoma (LS178Y), mastocytoma (P815), i.d., AJ/C57BL/6: sarcom (SA-1), melanoma, (B16), 5–8 mm	[75] [76]
	IL-12	Plasmid	i.d.		78% (vol)	BALB/c, renal cancer (Renca), s.c.	
	IL-12	HSV	i.tu.	7×10^5, 2× (helper)	94% (vol) 145% (surv)	BALB/c, colon cancer (CT-26), s.c., 5 mm	[87] [88]

	B7–1	tumor cells	MTX chemo	10^6, 2–4×	87% (red met) 200% (surv)	F344 rats, osteosarcoma (MSK-8G), ortho	[43]
	B7–1	Ad	i.tu.	10^9	no regression	C3H mice, melanoma (K 1735), s.c., 27 mm^3	[12]
	B7	tumor cells	i.p.		60% (cures)	C57/BL6, lymphoma (EL4), s.c.	
	B7–1	tumor cells	s.c.	5×10^6	13% (cures) n.s. (surv)	A/J, neuroblastoma (N-2a), s.c., 3–7 mm	[22] [45]
	Tumor peptides	Dendritic cells	i.v.	$3–5 \times 10^5$, several times	MCA205 82% (vol) TS/A > 50% (vol) C3 100% (cures)	C57BL/6, fibrosarcoma (MCA205), C3, i.d. BALB/c, breast cancer (TS/A), i.d.	[103]
	gp100	rVV generated T cells	i.v., rhIL-2 i.p.	1×10^7	96% (number of metastases)	C57BL/6, melanoma (B16), i.v.	[69]
Combinations	p16+p53	Ad	i.tu.	6×10^9, 2×	84% (vol)	nude, HCC, (HuH7) s.c.	
	p21+MHC class I, H-2Kb	Ad	i.tu.	2×10^8, 7×	62,5% (cures)	BALB/c, renal cancer (Renca), s.c., 4 mm^2	[78] [67]
	HSV-tk+IL-2	Ad	i.tu.	2.5×10^8	87% (area)	Lewis lung cancer (LL2) in liver, 4–5 mm	
	CD+GM-CSF	Ad		10^9, 2×	79% (vol)	C57BL/6, melanoma (B16F10), s.c.	[52] [15]
	IL-2+TNF	Plasmid	s.c.	5×10^6, 6×	growth retardation	C57/BL6, lung cancer (Lewis lung cancer carcinoma), s.c.	[66]
	IL-2, IFN-γ, GM-CSF,	tumor cells	i.d.	10^6, 3×	60% (cures) (s.c.) no (cures), surv benefit (orth)	Copenhagen rats, prostate cancer, (R3327-MatLyLu), s.c., ortho	[92]
	IL-2, γIFN	tumor cells	i.p.	1×10^6, 4×	IL-2 53% (number of metastases) γIFN 81% (number of metastases) no add. effect	C57/BL6, melanoma (B16), i.v.	[1]
	B7–1, MHC class II	tumor cells	i.p.	10^6, 1×	60% (cures)	A/J mice, sarcoma (Sal/N), s.c., 2–5 mm	[7]
	B7–1, GM-CSF, IL-12	tumor cells	i.d.	1×10^6, 3×	IL-12 30% (cures), 70% (vol) B7–1, GM-CSF no eff.	C57BL/6, lung cancer (LLC), i.d.	[83]
	B7–1, IL-12	Fibroblasts	peri.tu.	$1–2 \times 10^6$, 2/3×	55% (vol)	BALB/c, breast cancer (TS/A), i.d., 25–35 mm^2	[104]
	B7–1, IL-12	tumor cells	s.c.	5×10^6, weekly	IL-2 80% (cures) B7–1 no impact	DBA/2, mastocytoma (P1.HTR.C), s.c.	[35]

(*Continued*)

Table 2 Continued

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
	B7–1, B7–2	tumor cells	s.c.	10^6, 6×	RMA: 40% (cures) TS/A (B7–2): 22% (cures), TS/A (B7–1): no (cures) B16 no reponse	BALB/c, C57BL/6, lymphoma (RMA T), breast cancer (TS/A), melanoma (B16.F1) s.c.	[59]
	GM-CSF, IL-4	tumor cells	s.c.	3×10^6, d5	GM-CSF: 90 IL-4: 170% (surv)	BALB/c, lymphoma (A20), i.v.	[55]
	GM-CSF+IFN-γ	tumor cells	s.c.	10^6	145% (surv)	A/J, neuroblastoma (neuro 2a), ortho	[9]
	IFN-γ, IL-4	monocyte/ macroph-age cell line (J774A.1)	i.tu.	$2–4 \times 10^6$	IL-4 or IFN-gamma: 50% IL-4+ IFN-gamma: 25% (vol)	C57BL/6, melanoma (B16), s.c.	[65]

Abbreviations:
i.p.: intraperitoneal, i.tu.: intratumoral, i.pleu.: intrapleural, i.port.: intraportal, i.a.: intra-arterial, s.c.: subcutaneous, i.cr.: intracranial, i.d.: intradermal, ortho: orthotopic (typical site of primary tumor, HCC: Hepatocellular carcinoma, Ad: adenovirus, Retro: retrovirus, Retro-VPC: retrovirus vector producer cells, VV: Vaccinia Virus Lip: liposomes, HSV: herpes simplex virus, Lenti: Lentivirus, vol: volume, surv: survival.

[a]*Note*: References found within this table are located in a special list of References found on pg. 578.

[1] reduction of tumor weight or volume in percent of untreated control

[2] increase in survival in percent of the control

[3] the amount of DNA is indicated

[4] cures: tumor-free animals and long-term survivers

[5] mice if not stated otherwise

Table 3 Cancer Gene Therapy, In Vivo Gene Transfer, Preclinical Studies, 1999–2002—First Descriptions of New Principles

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
AntiOncogenes							
	HER-2/neu ribozyme	Ad	i.tu	10 MOI, 5×	BT-474 76% (vol) MCF-7 91%	nude, breast cancer (BT-474, MCF-7), s.c., 1 mm^3	[1]
	K-ras ribozyme	Ad	i.tu	2×10^9, 5×	39%[2] (1 inject) 71% compl. tum regress. (5 inject)	nude mice, adenocarcinoma (NCI H441), s.c.	[2]
	anti TGF-α	Lip	i.tu	50 μg DNA, 9×	76% (vol)	nude, head and neck squamous carcinoma (HNSCC), s.c., ø 2 mm	[3]
	anti HMGI(Y)	Ad	peri tu., i.p.	5×10^7 pfu, 4× (peri tu) 5×10^8 pfu (i.p.)	peri tu 66% (vol) i.p. 41% (vol)	nude, thyroid anaplastic carcinoma (ARO), s.c.	[4]
	anti c-myc	Ad	i.tu.		68,9% tumor growth	nude, gastric carcinoma (SGC7901), s.c.,	
	anti-uPAR	Ad	i.tu	5×10^9, 5×	85% (vol)	nude, glioblastoma (U87-MG), s.c., ø 4–5 mm	[5]
	N116Y ras mutant	Ad	tr.ur.	10^9 pfu, 3×	80% size 47% number of tumor masses	KSN nu, bladder cancer (KU-7 UMUC-2), ortho,	[6]
	anti CaSm	Ad			71% tumor growth, 175% incr. (surv.)	SCID-Bg, pancreatic cancer (AsPC-1), s.c.	[7]
	anti AFP	antisense oligos	i.tu	0.5 mg, 8×	38.6% (tumor weight) 73.8% (AFP content)	BALB/c, hepatoma (SMMC-7721), s.c.	[8]
	ErbB-2Δtk	Ad	i.tu.	10^9 pfu	52% compl. tum. regression	FVB, breast cancer (NDL); ortho, 75–150 mm^3	[9]
Tumor Suppressor Genes							
	MBP	VV	i.tu. s.c.	5×10^6 pfu, 4×	i.tu.-90%(vol) 75% (vol) decr. to init. size s.c.-46% (vol)	nude, colorectal carcinoma (SW1116), s.c.	[10]
	Gelsolin	Retro-VPC	i.tu.	10^5 CFU, 10×	>87% (vol) incr surv	nude, bladder cancer (UMUC-2 or DAB-1), s.c., 8.9 or 5.3 mm^3	[11]
	FHIT	Ad	i.tu	3×10^{10} 4×10^{10} pfu	88% (vol)	nu/nu, lung cancer (H1299 or A549), s.c., ø 5–10 mm	[12]
	Bax	Ad	i.p.	3×10^9 pfu, 2×	99% (nod.number)	nude, ovarian cancer (36M2), i.ab.,	[13]
	FasL	Ad	i.tu,	5×10^{10} vp, 2×	100% tumor regress	BALB/c, nude, SCID, mouse renal epithelial carcinoma (Renca), mouse colon carcinoma (CT26), s.c., ~0.5 cm	[14]

(*Continued*)

Table 3 Continued

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
	C-CAM1	Ad	i.tu.	$1,7\times10^9$ pfu, 3×	~90% (vol)	nude, prostate cancer (PC-3), s.c.,	[15]
	Stat3β	Plasmid electroin-jection	i.tu.	100μg, every 3–4 days	68–81% (vol) 80% tum régress >50% apoptose	C57BL, mouse melanoma (B16), s.c., ø 4–5 mm	[16]
	Pseudomonas exotoxin	Lip	i.tu.	20–40μl. DNA-lipid complex 4× MCF7, 3× A431	A431 75%(vol) MCF7 54% (vol) 30% decr. to init.gr.	nude, (A431 and MCF7), s.c., ø 0.5 cm	[17]
	P27-p16	Ad	i.tu	1.25×10^{11} vp, 3×	85% (vol) 50% vs. 0% long-term surv	BALB/c, prostate cancer (DU-145, PC-3), s.c., 30 mm^3	[18]
	CD66a	Ad	i.tu.	2×10^9 pfu, 3×	80% (vol)	nude, prostate cancer (DU145), s.c., 15 mm^3	[19]
	caspase-8	Ad	i.tu.	0.5×10^{10} pfu, 4×	72% (vol)	Balb/c, glioma (U251), s.c., ø 5–6 mm	[20]
	TNF-α + radiation	polyamin	s.c. (i.tu,) ?	50μg DNA, 3×	46% (vol)	nude, rat glioma (C6), s.c.,	[21]
	hFlex-TRAIL	plasmid	i.v.	10μg, 2×	83% vol	SCID mice, mammary adenocarcinoma (MDA-231), s.c.,	[22]
	RIZ1	Ad	i.tu. peritu	8×10^{10}, 4×	65% vol	nu/nu, coloteral carcinoma (HCT116), s.c., ø 0.5 cm	[23]
	TRAIL	Ad	i.tu	6×10^{10}, 3×	76% (vol)	nude, colon carcinoma (DLD-1), s.c., ø 0.5 cm	[24]
	hRAD50	Lip	i.tu	2μg DNA, 2×	78% complete tumor regression 83% surv.	Sprague-Dawley rats, rat mammary adenocarcinoma (RBA), s.c., ø < 1 cm	[25]
	Caspase-9	Ad	i.tu.	10^9 pfu	43% surv., tumor growth suppression	BALB/c, prostate adenocarcinoma (LNCaP), s.c., 58–98 mm^3	[26]
	mut.survivin	Ad	i.tu.	5×10^8, GFU/site ×3, 2×	93% (vol) 60% vs. 0% (long-term surv)	SCID, breast carcinoma (MCF-7), s.c., 100–150 mm^3	[27]
	TH	Ad	tr.cr.	10^8 iu	49% pituitary growth reduction	Buffalo rats, estrogen induced lactotroph hyperplasia within AP gland	[28]
	Connexin 26	Ad	i.tu.	5×10^9 pfu/tum 3×	UM-UC-3 50% (vol), +cisplatin 97% (vol) UM-UC-14 66% (vol), +cisplatin 80% (vol)	nude, bladder cancer (UM-UC-3, UM-UC-14), s.c., ø 5 mm	[29]

Vpr	Lenti	i.tu.	10^6pfu	> 40% compl. tumor regres. ~80% (vol)	C3H mice, squamous carcinoma (AT-84), s.c., 100–200mm^3, 600–1000 mm^3	[30]
Bik	Lip	i.v.	15μg DNA, 6×	57,6%(vol) MDA-MB-231 42%(vol) MDA-MB-468 200% incr (surv)	nude, breast cancer (MDA-MB-231, MDA-MB-468), ortho, 4×4 mm	[31]
FADD	plasmid	i.tu.	50μg, 7×	40% (vol)^2 – init. tum.size 90% (vol)-control	BALB/c, glioma (U87-MG), s.c., 50–70 mm^3	[32]
RA-538	Ad	i.tu.	10^9 pfu, 3×	60,66% tumor growth	nude, gastric carcinoma (SGC7901), s.c., ø 5mm	[33]
DT-A	Ad	i.tu.	10^9 vp	LNCaP- 80% vs. 0% (long-term surv) 70–99% (vol); DU145 – not efficient	nude, prostate cancer (LNCaP, DU145), s.c., 300 mm^3	[34]
N5	Ad	i.tu.	10^8 pfu, 3×	SKOV3-IP1 97%(vol) FG 82%(vol) 100% (met)	nude, ovarian carcinoma(SKOV3-IP1) or pancreatic adenocarcinoma(FG), s.c., ortho., 40–50 mm^3	[35]
3p21.3 genes—101F6, FUS1, NPRL2, HYAL2 HYAL1	Ad, Protamin-AD	i.tu; i.v.-	5×10^{10} vp/tum, 3×-tum. gr.mod 3×10^{10} vp/tum, 3×-met. mod.	HYAL2 HYAL1-no tum.gr. suppr., A549 cells 101F6–92%vol NPR2–77%vol FUS1–55%vol; H1299cells 101F6–80%vol NPR2–51%vol FUS1–71%vol; 101F6, FUS1, NPRL2–80% met.num., HYAL2–70% met.num.	nude, 3p21.3 120-kb region-deficient (homozygous) H1299 and A549, s.c., ø 0.5cm; A549, ortho—met.mod.	[36]

(*Continued*)

Table 3 Continued

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
	decorin	Ad	i.tu.	7×10^7 pfu, 5–7×	WiDr-67%vol A431–52%vol	nude, colon carcinoma (WiDr), squamous carcinoma (A431), s.c., ø 2–3mm	[37]
	maspin	Lip	i.tu. + i.v.	37.5mg/site, 2× +150mg/ mouse, 6×	28% (vol) 29% (tum.gr.rate) 55,5%(met)	FVB, mammary tumor (PyV MT), ortho, $< 0.5cm^3$	[38]
Prodrug activating Genes							
	PNP	VV	i.p.	10^6pfu	30% vs. 0% long-term surv	nu (C56BL/6), colon adenocarcinoma (MC-38), i.spl.	[39]
	UPRT + CD	Ad	i.tu.	$5\times10^7+ 5\times10^7$ pfu	UPRT alone—no effect, CD-67% (vol), combined—94% (vol)	BALB/cAnCrj-nu/nu, colon cancer (HT29), s.c.	[40]
	NTR	Ad	i.tu.	$2{,}5\times10^9$ -2×10^{10} vp	50–88% (treated tum.)	Balb/c nu mice, hepatocellular carcinoma (HepG2), s.c., 20–85mm^2	[41]
	NIS	Ad	i.tu.	9×10^7 pfu, 5×	84 ± 12%(vol)	BALB/c, prostate adenocarcinoma (LNCaP), s.c., 5×5 mm	[42]
Antiangiogenic genes							
	anti VEGF	Ad	i.tu.	10^9 pfu 4×	85% (vol)	nude, glioma (U-87 MG), s.c.,	[43]
	Endostatin	Polymer	i.m.	240 μg DNA, 2×	6-fold red met, 40% (vol)	BALB/c and C57BL, murine renal carcinoma, (Renca) and metastasic variant of LLC, s.c., $10mm^3$	[44]
	Angiostatin	Lip	i.tu.	2,9 mg DNA, 3×	36% (vol)	nude, breast cancer (MDA-MB-435 and CHO), ortho,	[45]
	Flt-1	Lip	i.p.	30 μg DNA, 4×	80% (vol) 50% vs 0% long-term surv	BALB/cAnCrj-nu/nu, gastric cancer (MKN45), i.p., ø 5mm	[46]

mATF	Ad	i.tu.	10^9 pfu, 5×10^9 pfu (LS174T)	MDA-MB-231 85% (vol) LLC 70% (vol) LS174T 75% (met.num.)	nude, C57BL/6, colon carcinoma (LS174T) met.mod.; breast carcinoma, (MDA-MB-231), Lewis lung carcinoma (LLC), s.c., 15–20 mm^3	[47]
RB2/p130	Retro	i.tu.	5×10^6/tum	81% red. of microvessels count	nude, lung adenocarcinoma (H23), glioblastoma (HJC Δ5 and HJC 12), s.c., 20mm^3	[47]
Proliferin	Ad	1×i.v. 3×i.tu.	10^9 IU, 4×	42% complete tumor rejection 40% vs. = % long-term surv	B6D2, melanoma (B16F10), s.c.	[48]
TSP-1	Lip	i.tu.	10μg, 3×	75% (vol)	Balb/c, prostate cancer (DU145), s.c., ø 0.5 cm	[49]
Flk1	Ad	i.v.	10^9 pfu	80% (vol) 74% (neovasc)	C57BL/6, Lewis Lung carcinoma (LLC), fibrosarcoma (T421), s.c., 100–150 mm^3, SCID, pancreatic carcinoma (BxPC3), 60mm^3	[50] [51]
NF-kappaB decoy ODN	antisense oligos	i.v.	30 μM, 3×	80% number of metastasis	C57BL/6, reticulosarcoma (M5076), i.v. (hep.met.mod)	[52]
MME	Lip	i.tu.	18mg, 4×	77% (vol) 40% microvessels	BALB/c, mouse colon cancer (CT-26), s.c.	[53]
Vasostatin	Plasm id	i.m.	100 μg, 8×	80% vol (MethA), 72% vol (LL/2c), 70% vs. 0% (long-term surv) 67% blood vessel length 68% clock hours 79% area of neovascularization	BALB/c, fibrosarcoma (MethA); C57BL/6, Lewis Lung carcinoma (LL/2c),	[54]

(*Continued*)

Table 3 Continued

Strategy	Transgene	Vector	Route	Dose	Efficacy	Animal model, organ, cell line, site, pretreatment size	Reference[a]
	ATF-BPTI	Ad	i.tu. i.v.	10^{10} iu, 1×(i.v.) 3×(i.tu.)	i.v. L44-86% (vol), i.tu. L42-72%(vol), i.tu. L44-no.inh., L42,-50% met	Wag/Rij rats, bronchial squamous carcinoma (L42); Brown Norway rats, adenocarcinoma (L44), s.c., 8 × 8 × 8 mm;. i.v. for metast. model.	[55]
	TIMP-1 TIMP-2	Ad	i.p.	10^9, 3×	75% 85% (vol)	nude, pancreas, peritoneum	[56]
	TIMP-3	Ad	i.tu.	10^9, 3×	marked (size)	Scid, melanoma, (2058), 50–100 mm^2	[57]
Combinations							
	anti-telomerase RNA, p53	Ad, lipofectamin	i.tu.	1nmol, 6×10^7 pfu, 3×	66% (vol)- to initial size 84% (vol)- to contr.	Balb/c, glioma (U251-MG), s.c., 32–40mm^3	[58]
	p53, anti cyclin D1	Ad	i.tu.	5×10^8, 2×	57% of tum. vanished	SCID, melanoma (1205), s.c., 75 mm^3	[59]
	TRAIL, Bax	Ad	i.p.	6×10^{10}	48% surv., >99%(vol), no tumor-free animals	nude, ovarian cancer (SKOV3), i.p.,	[60]
	EMAP-II + TNF-α	VV	i.v.	10^6 pfu+ 7μg×2 DNA	70% (vol)	NCR-nu, melanoma (Pmel), s.c., 300mm^3	[61]
Oncolytic Viruses		mut. HSV	i.tu.	10^7 pfu, 3×	hs766t 96%(vol) HLB147 75% (vol)	nude, pancreatic cancer (hs766t and HTB147), s.c., 22 mm^3 (hs766t), 30 mm^3 (HTB147)	[62]
	CYP2B1	HSV	i.tu.	10^8 pfu, 4×	90% (vol)	nude, glioma (U87ΔEGFR), s.c., 200mm^3	

Abbreviations: See the footnote for Table 2 on page 556.

[a]*Note*: References found within this table are located in a special list of References found on pg. 582.

Table 4 Cancer Gene Therapy, In Vivo Gene Transfer—Published Clinical Studies Until 2002

Transgene	Vector	Route	Dose	Tumor type	Indication of gene transfer	Clinical efficacy	Lab findings efficacy	Toxicity	References[a]
HLA B7	Lip	i.tu.		advanced melanoma	B7 protein in tumor	regression of nodules	immune response	no complications	Nabel GJ, 1993 [1]
CD/HSV-1TK	Ad	i.tu.	10^{10}–10^{12} particles	prostate cancer	transgene expression confirmed	decrease in serum prostate-specific antigen in 63% of patients		adverse events observed were mild or moderate	Freytag SO, 2002 [2]
HSV-tk	Ad			prostate cancer	vector DNA detectable	increases in PSA doubling time, PSA-reduction, and return to initial PSA-level, and activated CD8(+) T cells			Miles BJ, 2001 [3]
none	Vaccinia virus	i.tu.	3×10^3 pfu	muscle invasive transitional cell carcinoma	efficient infection and transfection	75% of patients stayed free of disease for 4 years	significant inflammatory infiltration	mild dysuria	Gomella LG, 2001 [4]
HSV-tk	Ad	i.tu.	1×10^{10}–1×10^{13} particles	metastatic colorectal adenocarcinoma			hepatic necroinflammation	fever, thrombocytopenia, leucopenia	Sung MW, 2001 [5]
HLA-B7, β2-microglobulin	Lip	i.tu.	$6 \times 10\mu g$	metastatic melanoma		regression of injected lesion in 18% of patients		ecchymosis, pruritus, pneumothoraces	Stopeck AT, 2001 [6]
none	ONYX-015	i.tu.	$2*10^{11}$ particles	squamous cell carcinoma	circulating genomes and replication	14% partial to complete regression, 41% stable disease, 45% progressive disease		mild to moderate fever and injection site pain	Nemunaitis J, 2001 [7]
HSV-TK	Ad	i.p.	1×10^9 to 1×10^{11} pfu	ovarian cancer	Transgene DNA and RNA present	7% response, 38% stable disease, 61% progressive disease		abdominal pain, gastrointestinal symptoms	Alvarez RD, 2000 [8]
p53+ cisplatin	Ad	i.tu.	10^6 to 10^{11} pfu	lung cancer	mRNA detectable	stable disease (71%), partial response (8%), progression (16%)		fever	Nemunaitis J, 2000 [9]
IFN-γ	Retro	i.tu.	10^7 pfu 6 cycles or once	metastatic melanoma		Stable disease (62%), complete response (38%), Response (11%) one injection	immune response against MAGE-A1, tyrosinase Ab, MAA	not reported	Fujii S, 2000 [10]
none	ONYX-015	i.tu.	10^{10} pfu+ cisplatin+ 5-fluorouracil	squamous cell carcinoma		Complete reponse (33%), partial response (33%), minor response (11%), stable disease (22%)		mucositis, constipation, fatigue, nausea, vomiting	Lamont JP, 2000 [11]
anti-erbB-2	Ad	i.p.	10^9–10^{11} pfu	ovarian cancer	expression detectable	38% response, 61% progressive disease		fever, pain, gastrointestinal symptoms	Alvarez RD, 2000 [12]

(*Continued*)

Table 4 Continued

Transgene	Vector	Route	Dose	Tumor type	Indication of gene transfer	Clinical efficacy	Lab findings efficacy	Toxicity	References[a]
HSV-tk	Ad	i.tu.	$2*10^9$–$2*10^{12}$ particles	advanced recurrent malignant brain tumors		death within 10 month (77%) or 25 month (14%), survival longer than 29 months (7%)	necrosis, infiltration	central nervous system toxicity	Trask TW, 2000 [13]
wt p53	Ad	i.tu.	tumor size dependent	metastatic melanoma or breast cancer	mRNA detectable	stable disease (67%), progressive disease (33%)		mild reactions	Dummer R, 2000 [14]
HLA-B7	Lip	i.tu.	max 250μg DNA	renal cancer	gene product detectable	stable disease or progressive disease	no autoimmune reponse	no complications	Rini BI, 1999 [15]
HSV-tk	Retro	intracerebral		recurrent glioblastoma multiforme	vector DNA in leukocytes	marginal effect		transient neurological disorders	Shand N, 1999 [16]
IL-2	Lip	i.tu.	6*10–300μg	metastatic melanoma, renal cell carcinoma	plasmid detectable	response (86%)	CD8+ infiltration	mild constitutional symptoms to grade III toxicity	Galanis E, 1999 [17]
IL-2	Ad	s.c.	10^7–10^{10} pfu	melanoma or breast cancer	mRNA and vector sequences detectable	24% patients showed incomplete local tumor regression	tumor necrosis and lymphatic infiltration	local inflammation	Stewart AK, 1999 [18]
wt p53	Ad	i.tu.	10^6–10^{11} pfu	head and neck cancer	expression detectable	27% of patients stayed free of disease		fever, injection site pain	Clayman GL, 1999 [19]
IFN-γ	Retro	i.tu.	$1,5*10^8$ cfu	advanced melanoma	DNA-transduction detectable	feasiblity confirmed		no complications, no replication of vector	Nemunaitis J, 1999 [20]
IFN-gamma	Retro	i.tu.	multiple times max 10^7 pfu	advanced melanoma		53% stable disease, 47% elevated anti-tumor antibodies	antitumor immune response	Grade I/II events, no replication of vector	Nemunaitis J, 1999 [21]
HSV-tk	Ad	i.tu.	10^8–10^{11} infectious units	adenocarcinoma of the prostate	Vector detectable by PCR	objective response (17%)		Grade I/II events, no replication of vector	Herman JR, 1999 [22]
wt p53	Ad	i.tu.	10^6–10^{11} pfu	lung cancer	Vector DNA and RNA detectable by PCR	partial response(8%), stable disease(64%), progression (28%)	apoptosis detectable	Grade I/II events, one patient grade III	Swisher SG, 1999 [23]
wt p53	Ad	i.tu.		lung cancer	gene transfer detectable by RT-PCR and PCR	Stable(66%) or progressive disease(33%)		flu-like symptoms	Kauczor HU, 1999 [24]
HSV-tk	Retro	cells injected into surgical cavity		glioblastoma		25% of patients survived longer than 1 year, 1 patient free of disease		no complications	Klatzman D, 1998 [25]
HSV-tk	Retro	i.tu.	$7,6*10^8$ to $1,2 \times 10^9$ cells	metastatic melanoma	Transgene detectable by PCR	Limited long-time tumor response		Inflammatory skin reactions, fever	Klatzman D, 1998 [26]

HLA-B7	Lip	i.tu.		squamous cell carcinoma	HLA-B7 detectable	44% partial response	Apoptosis-induction if transgene detectable	no complications	Gleich LL, 1998 [27]
none	Onyx-015	i.tu.	$5*10^{10}$ pfu	recurrent squamous cell cancer	tumor-selective viral replication	Objective responses	Necrosis of tumor	acceptable	Khuri FR, 2000 [28]
wt p53	Ad	i.tu.	10^{7}–10^{10} pfu	lung cancer	RNA detectable	transient local disease control (66%)		no complications	Schuler M, 1998 [29]
wt p53	Ad	i.tu.		lung cancer	expression evident	potentially useful clinical responses		no complications	Roth JA, 1998 [30]
IL-12	Cells	s.c.	10^{5}–10^{7} cells	melanoma	strong secretion of IL-12	increased antitumor immune response (66%)		no complications	Sun Y, 1998 [31]
HSV-tk	Ad	pleural cavity	10^{9}–10^{12} pfu	mesothelioma	gene transfer shown		Strong immune responses	minimal side effects	Sterman DH, 1998 [32]
HLA-A2	Lip	i.tu.	up to 80 μg DNA	several	expression detectable	Complete (25%) and partial response (50%)	strong local responses	no significant side effects	Hui KM, 1997 [33]
HLA-B7	Lip	i.tu.	up to 250 μg DNA	metastatic malignant melanoma	expression detectable	significant responses (60%)			Waddill W 3rd, 1997 [34]
HLA-B7	Lip	i.tu.	up to 250 μg DNA	colorectal carcinoma	plasmid, mRNA, Protein detectable			no serious toxicity	Rubin J, 1997 [35]
IL-2	Retro	chest cavity	$1–6*10^{10}$ cells	lung cancer		tumor size decreased in 1 of 6 patients		minimal fever	Tan Y, 1996 [36]
none	Newcastle Disease Virus	i.v. Intravascular	up to $12*10^{10}$ pfu	advanced solid cancers	replication of PV701	Objective responses	lymphoplasmacytic infiltrate	flu-like symptoms	Pecora AL, 2002 [37]
none	ONYX-015		$2*10^{8}$–$2*10^{12}$ particles	gastrointestinal carcinoma	viral replication detectable	objective response in combination with chemotherapy	increasing antibody titers	fever, rigors and fatigue	Reid T, 2001 [38]

Abbreviations: See the footnote for Table 2 on pg. 556.

[a]*Note*: References found within this table are located in a special list of References found on pg. 583.

stalled viruses; the easy access to manifestations of head and neck cancer for the intratumoral transfer of replication-competent vectors; and the good infectability of HCC by adenoviral vectors or of glioblastoma by HSV (which is a neurotrophic virus by nature). Particularly encouraging results have been obtained in many immuno-gene therapeutic trials that do not depend as heavily on efficacy of gene transfer as nonimmunological approaches. The sometimes astonishing modulatibility of vectors gives rise to the hope that cancers that are less suited for cancer gene therapy will also have particular vectors designed against them.

Although most clinical studies were designed as phase I or phase I/II trials with the aim of dose-finding and toxicity evaluation, the lack of a real breakthrough in clinical efficacy has been disappointing—at least for those who had envisioned a very rapid development of gene therapy into a routine treatment modality. The discrepancy between excellent preclinical data and the clinical outcome has been particularly difficult to understand, especially for the public. There are several reasons for this discrepancy. First of all, animal models are artificial models that have inherent simplifications as compared to the clinical situation. A well-known argument is that animal tumors are usually developed from 1 fairly homogenous cell line, whereas human tumors are often very heterogeneous, though they have also originated from a single clone. Consequently, a strategy that targets one specific molecular event in an animal tumor may only be effective against a fraction of tumor cells in a patient. But there are less sophisticated reasons such as the mere difference in size of tumors between a rodent and a human. This may also dramatically affect clinical outcome. Another explanation is that dose-limiting toxicity has not been reached in most of the trials performed so far. In addition, mice are probably more robust and will probably tolerate higher doses. It is evident that some kinds of treatments well tolerated in mice would hardly ever be considered for humans.

Other setbacks for cancer gene therapy have included the first severe adverse events in gene therapy: the death of a young man in a trial for the metabolic disorder OTC and the very recent occurrence of a case of leukemia in what was a very successful trial for severe combined immunodeficiency up to that point.

All these considerations lead to the conclusion that the therapeutic window of most cancer gene therapeutic strategies is apparently still too narrow to allow prominent antitumor efficacy. However, nearly all kinds of medical therapy have been confronted with this problem during their development into routine clinical applications. This therapeutic window can be broadened by either creating less toxic medicines, which allows one to increase the dose, or by creating more efficient medicines, which allows one to obtain efficacy without the necessity of increasing the dose. Both avenues have been followed in cancer gene therapy. An example of a less toxic vector is HD-Ad as compared to first generation Ad. An example of a more efficient therapeutic principle is the combination of conventional, suicide-gene strategies with replication-competent viral vectors to increase the efficacy of gene transfer. A third strategy, which allows a reduction in the dose without losing efficacy, is vector targeting—either in the form of transductional or transcriptional targeting. This extremely promising approach will eventually reveal the real potential of gene therapy: the design of intelligent drugs that are capable of overcoming intelligent cancer.

But won't the design of small molecular drugs be intelligent enough to solve the cancer problem? Do we need gene therapy if small molecules can do the job? Especially if one considers the recent genomics-related, dramatic increase in knowledge about potential drug targets? Maybe in some cases. Admittedly, the use of a vehicle to transfer a gene into the human body is less direct and usually more complicated than conventional methods of cancer therapy. However, the interposition of several steps between the application of an active substance and the generation of an observable effect allows an unusual high degree of freedom of regulation. In cancer gene therapy, one can comply with the biology and especially the weak spots of the cancer cells to a degree never before seen. Fortunately, nature seems to be predictable to a certain degree, which makes such complex therapeutic strategies possible. Since we are still far away from such a tight adaptation of our vectors and transgenes to the specificities of the cancer cells, it comes as no surprise that to date clinical efficacy of cancer gene therapy has not been convincing. However, the potential fruitfulness of a new method cannot be judged by its initial success but rather by the general limitations of the whole concept. Since in the area of cancer gene therapy such limitations have not yet been seen, a broad breakthrough in therapeutic efficacy will most likely be a matter of time.

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28

Overcoming Barriers to Efficient Airway Epithelial Gene Transfer

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I. INTRODUCTION

The identification of human genes linked to clinical disease has raised hopes for the development of genetic therapies for many inherited and acquired diseases. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene lead to a common inherited disorder, cystic fibrosis (CF), with a high morbidity and mortality that makes it an attractive target for gene therapy. The autosomal inheritance pattern of this monogenic disorder in which heterozygotes are phenotypically normal, combined with the relatively large numbers of patients available for clinical studies, has lead to the establishment of CF as the prototypical disease for investigation of gene therapy in the lung.

Because CF heterozygotes exhibit a normal phenotype, introduction of a single wild-type (normal) copy of the gene into defective CF epithelial cells should restore the normal phenotype. Restoration of CFTR-mediated Cl^- transport function following introduction of wild-type *CFTR* into CF airway epithelial cells in vitro using retrovirus, vaccinia virus, liposomes, and adeno-associated virus (AAV) and adenovirus (Ad) vectors is consistent with this concept (1–5). These studies have also established the feasibility of gene therapy for CF.

The cellular targets for CF gene therapy in humans have not been clearly elucidated. The site where CF lung disease begins remains controversial with both the superficial columnar epithelial cells lining the lumen of the small airways and the serous cells of submucosal glands having been identified as potential sites. Clinical data tends to support the theory that the disease begins in the small airways (6,7), whereas the submucosal glands are the predominant site of CFTR expression (8). Where the disease begins is relevant because luminal (airway) delivery of gene transfer vectors primarily targets the superficial columnar airway epithelium, whereas intravenous (blood) delivery may be required to target the submucosal glands and basal cells in the airway.

Stem cell niches may also exist within the lung that may ultimately be the targets for airway gene transfer. These stem cell niches appear to consist of subpopulations of submucosal gland duct cells in the proximal airways and subpopulations of clara cell and/or clara cell secretory protein-expressing cells in the distal airway (9,10).

Following initial in vitro complementation studies, investigators rapidly moved to clinical safety and efficacy trials of gene transfer vectors delivered by luminal application to the airways of CF patients. Although some evidence for gene transfer was detected, the efficiency and efficacy of gene transfer failed to meet expectations and did not fully correct the known functional defects ascribed to this disorder (11–25). This failure forced exploration of potential barriers to gene transfer in airways and stimulated efforts to develop strategies to overcome these barriers. In this chapter, we discuss the barriers that have become apparent from preclinical and human studies, and explore current strategies to overcome these barriers to luminal airway gene transfer.

II. VECTORS FOR LUNG GENE TRANSFER

Naturally occurring viruses are attractive for gene transfer vectors because they have evolved the ability to introduce their own viral nucleic acid (DNA or RNA) into the host cell nucleus, leading to expression of viral genes that promote viral replication. This property has been exploited for the develop-

ment of viral vectors that introduce and express therapeutic genes (cDNAs) in lieu of their viral structural genes, which have been deleted. Nonviral vectors based on cationic lipids and poly-L-lysine-based polymers have also been developed. The characteristic features, advantages, and disadvantages of Ad, AAV, and nonviral vectors are summarized in Table 1.

Twenty-two phase I and II clinical gene transfer safety and efficacy trials in CF patients have been registered with the Office of Biotechnology Activities at the National Institutes of Health (http://www4.od.nih.gov/oba/rac/clinicaltrial.htm), and many others have been performed in the United Kingdom, France, and Italy. Most of these protocols involve direct delivery of CFTR vectors to nasal or lower airway epithelia, whereas the remaining trials deliver an alpha-1 antitrypsin lipoplex to the nasal airway and AdCFTR vectors intradermally. Initial phase I trials evaluated single administration of Ad-*CFTR* vectors to the nasal and/or lower airway epithelia of CF patients. Subsequent trials of Ad vectors have evaluated the feasibility of repetitive dosing and the safety and efficacy of aerosolized Ad vector administration. Trials of cationic liposomes complexed to CFTR plasmid DNA (lipoplexes) have increased in number, and in the past 2 to 3 years, trials of aerosolized AAV-mediated gene transfer to CF airways have been initiated. One trial of stabilized CFTR polyplexes (poly-L-lysine complexed to CFTR plasmid DNA) has also been registered, but clinical data have not been reported for this trial.

The preliminary results of the aerosolized AAV2 trial are perhaps noteworthy (see www.cff.org) (26). This study is a randomized, double-blinded, placebo-controlled trial of repetitive aerosol administration of an AAVCFTR vector (tgAAVCF) to 37 subjects (17 placebo, 20 aerosolized tgAAVCF) with mild lung disease. Subjects received 3 doses of 10^{13} DNase-resistant tgAAVCF particles at 30-day intervals. Preliminary results suggested a statistically significant improvement in FEV1 at 30 days with decreased IL-8 levels in subjected receiving aerosolized vector as compared with placebo controls. These data have not yet been published, but have stimulated initial planning for a larger trial to assess efficacy.

Despite these data, the general consensus from the literature would suggest that (1) in vivo gene transfer efficiency and efficacy has been inadequate to correct the functional properties ascribed to this disorder, (2) the inefficiency of all vectors tested would suggest that common barriers to gene transfer may exist in addition to vector-specific barriers, and (3) immune or inflammatory reactions may also limit gene transfer, particularly with regard to Ad vectors and some lipoplexes. In the next section, we focus on barriers in the airway lumen, at the cell surface, and intracellular barriers that limit binding, entry, and gene expression. Although safety is a major concern, immune barriers (e.g., neutralizing antibodies and immune/inflammatory responses to the administered vector that limit transgene expression) are not discussed in any detail because the major limitation to CF airway gene transfer is low efficiency.

III. BARRIERS TO AIRWAY GENE TRANSFER

Several barriers to luminal airway gene transfer have been described. These include contents within the airway lumen, such as mucus, inflammatory cells, or bacteria, and cell-associated components of the airway surface liquid (ASL) and glycocalyx. These components are referred to collectively as nonspecific barriers. Cellular barriers to airway gene transfer that are specific for a particular gene transfer vector (vector-

Table 1 Vectors for Airway Gene Transfer

Gene transfer vectors	cDNA insert size (kbp)	Duration of expression	Transduction of nondividing cells	Immune/inflammatory response	Risk of insertional mutagenesis
Adenovirus					
First Second generation	7–8	Transient	Yes	Humoral/cell	Minimal
Helper dependent	>30	Transient[a]	Yes	Humoral/cell	Minimal
Adeno-associated virus	4.5	Long term	Yes	Humoral	Low
Retrovirus					
MLV[b]	7	Long term	No	None	Yes[c]
Lentivirus[d]	>7	Long term	Yes	None	Yes
Cationic liposomes	>10	Transient	Yes	Inflammatory	Minimal
Polymers	>10	Transient	Yes	Unknown	Minimal

[a] Transient expression from helper-dependent Ad vectors can be prolonged.
[b] Derived from MLC.
[c] Two cases of leukemia have been reported in a human trial of X-linked severe combined immunodeficiency in which human stem cells were transduced with an MLV vector containing a gamma-C transgene under the control of the retroviral LTR.
[d] Derived from human immunodeficiency virus, equine infectious anemia virus, or feline leukemia virus. Experience with helper-dependent adenoviral vectors is limited.

specific barriers) include factors affecting cell binding and entry, nuclear translocation, and factors limiting transgene expression post nuclear entry. These barriers combine to make in vivo gene transfer to human airways inefficient.

A. Nonspecific Barriers to Gene Transfer

1. Luminal Contents

The current understanding of CF disease pathogenesis is based on mutant CFTR-mediating defective ion transport that reduces ASL height and volume with resultant abnormal secretions, ineffective mucociliary clearance, bacterial proliferation with multiresistant organisms and chronic inflammation, bronchiectasis, and premature death (27–30). An inflammatory response characterized by a massive influx of neutrophils, which releases oxidants and proteolytic enzymes promoting tissue injury and bronchiectasis, is a hallmark of this disorder. The influx of neutrophils also increases the load of DNA and actin in the airway secretions of the CF lung, leading to a markedly increased sputum viscosity with markedly elevated levels of the proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 that perpetuate the inflammatory response (31). This exaggerated inflammatory response with abnormal secretions, inflammation, and pseudomonas colonization is established early in life, often in the first year (32–35). Because current therapies fail to eradicate the chronic pulmonary infection and inflammation of the CF lung, luminal gene transfer approaches targeting the superficial airway epithelium must overcome the inflammatory response in order to gain vector access to the airway epithelium.

Stern et al. investigated the effect of fresh sputum obtained from CF patients on gene transfer to primary airway cells and to CF cell lines in vitro(36). A dose-dependent inhibition of gene transfer to COS-7, 16HBE14o$^-$, and 2-CFSMEO cells mediated by the cationic liposome DC-Chol/DOPE and an Ad-*lacZ* vector was detected in the presence of ultraviolet light-sterilized CF sputum. The effects of CF sputum on liposomal gene transfer were partially reversible with recombinant DNAse (rDNAse) pretreatment and completely reversible when rDNAse pretreatment preceded Ad gene transfer. Application of genomic DNA to cultures prior to transduction with DC-Chol/DOPE or an Ad-*lacZ* vector simulated the inhibitory effects of sputum on gene transfer suggesting that excessive DNA was a major contibutor to the inhibitory effects of noninfectious sputum components on gene transfer mediated by cationic liposomes and adenoviruses. Pretreatment with other mucolytic agents, including nacystelyn, lysine, n-acetylcysteine, and rAlginase, failed to increase liposomal or Ad gene transfer efficiency. However, in an ex vivo sheep tracheal model, pretreatment with n- acetylcysteine, nacystelyn, or mucus depletion overcame the effects of mucus on gene transfer mediated by L-polyethylenimine (L-PEI) polyplexes and p-ethyl-dimyristoylphosphatidyl choline (EDMPC) lipoplexes (37). Thus, both CF and normal mucus may have inhibitory effects on gene transfer.

Perricone et al. evaluated the effects of the CF sputum on Ad vector infectivity (38). Sputum was collected from CF patients with acute exacerbations receiving antibiotics and rDNAse, and separated into aqueous (sol) and gel components by ultracentrifugation. Pooled CF sol samples inhibited Ad gene transfer to fetal rat tracheal and normal human bronchial epithelial cells. Subsequent studies demonstrated that Ad-specific antibodies within the sol of CF sputum inhibited Ad gene transfer. Despite the presence of neutrophil elastase (NE) within the CF sol, pretreatment of CF sol with proteinase inhibitors did not prevent CF sol-induced inhibition of Ad gene transfer.

The technique of bronchoalveolar lavage (BAL) harvests luminal contents from the airways and the alveolar region of the lung. It may also harvest soluble components from the airway and alveolar surfaces. BAL fluid from CF patients has been shown to inhibit AAV gene transfer to IB3-1 cells and C12 cells in vitro (39). This inhibitory effect was reversible when CF BAL fluid was incubated with alpha 1-antitrypsin (α_1AT), and correlated with markedly elevated levels of NE and human neutrophil peptide (HNP) in BAL fluid from CF subjects. However, studies with purified HNP and NE demonstrated that HNP, rather than NE, mediated the major inhibitory effect on AAV gene transfer. Thus, HNP in BAL fluid from CF patients may have inhibitory effects on AAV gene transfer.

In contrast, Rooney et al. demonstrated that non-CF, rather than CF BAL fluid, inhibited Ad5 gene transfer to well-differentiated (WD) human airway epithelial (HAE) cells expressing glycosylphosphatidyl inositol (GPI)-coxsackie and adenoviral serotype 2/5 receptor (CAR) on the apical membrane (40). This effect was caused by the presence of IgG and I gA antibodies in BAL fluid, which could be removed by immunoprecipitation with staphylococcal A protein with restoration of Ad gene transfer efficiency. Unlike Ad5, neither CF nor non-CF BAL fluid had any effect on gene transfer mediated by an AAV5 vector.

Pseudomonas aeruginosa-induced bronchopulmonary inflammation may also inhibit Ad-mediated gene transfer (41). In an animal model of chronic bronchopulmonary infection, in which mice inoculated with pseudomonas aeruginosa-laden agarose beads develop bronchitis, bronchopneumonia, bronchiectasis, mucus plugging, and alveolar exudate with acute and chronic inflammatory cells, a greater than 2-fold reduction in gene transfer efficiency was detected following nasal instillation of an Ad-*lacZ* vector, as compared with mice that had sterile beads or Ad vector alone instilled. Similarly, the efficiency of Ad-*lacZ* gene transfer to nasal airways of pseudomonas (PAO1 strain)-infected mice was reduced by 10-fold as compared with noninfected nasal airways (42). Thus, the inflammatory milieu induced pseudomonas is a formidable barrier to transduction.

Airway and alveolar macrophages may also serve as barriers. Worgall et al. (43) used Ad-specific probes and Southern blot analysis to demonstrate a 70% loss of Ad-*lacZ* genomes within 24 h in both immunocompetent and immunodeficient mice 24 h following transtracheal administration of an Ad-*lacZ* vector. Pretreatment of murine lungs with liposomes containing dichloromethylene biphosphonate to eliminate macro-

phages, followed by administration of an Ad-*lacZ* vector, resulted in a significant increase in lung DNA and subsequent β-galactosidase expression. In vitro studies in cultured human alveolar macrophages demonstrated rapid loss of Ad vector genomes consistent with the in vivo data.

Retrovirus-mediated gene transfer to airway epithelia (44) may also be inhibited by alveolar macrophages. Transduction of human airway epithelial cells by an amphotropic enveloped retroviral vector was inhibited ~40% by alveolar macrophages and by more than 60% by lipopolysaccharide (LPS)-activated alveolar macrophages (35). Incubation of macrophages with dexamethasone (1 μM) partially reversed this inhibition of retroviral transduction. Rapid uptake of labeled vector into vesicles of macrophages was associated with loss of DNA within 24 h consistent with rapid degradation, rather than rapid transduction, of alveolar macrophages. These data suggest that macrophages can play a significant role in inhibiting in vivo gene transfer to lung epithelia.

2. Cell Surface Components

Components of the ASL and the glycocalyx may also have inhibitory effects on airway gene transfer. The effect of ASL on airway gene transfer has not been extensively evaluated. McCray and coworkers (44) demonstrated that airway surface liquid from WD HAE cell cultures harvested in a small volume of distilled water failed to inhibit retroviral transduction to naive airway cells in vitro. Airway surface fluid, obtained by washing the surfaces of WD airway cell cultures and bronchial xenografts, also had no effect on transduction mediated by AAV-2 vectors (45). Although the samples of the airway surface fluid used in these studies were dilute, these data would suggest that insignificant levels of vector inhibitory substances are present within the soluble components of airway surface fluid.

The glycocalyx is a complex structure on the apical surfaces of airways consisting of complex carbohydrate moieties (sugars), glycolipids, and glycoproteins. At least 5 mucin glycoproteins have been localized to airway surfaces: MUC1 and MUC4, which are tethered mucins and MUC2, MUC5AC, and MUC5B, which are secreted mucines that adhere to the cell surface (46–50). Arcasoy and colleagues (49,51) demonstrated that overexpression of the mucin MUC1 reduced Ad-mediated gene transfer to MDCK and bronchial epithelial cells that could be overcome by removal of sialic acid residues from the apical surface by neuraminidase pretreatment (49,51). Two groups have recently taken advantage of GPI-linked CAR to more carefully evaluate the role of the glycocalyx as a barrier to gene transfer. Overexpression of GPI-linked CAR in MDCK cells and HAE cells results in localization of CAR predominantly to the apical membrane of polarized cells (52,53). Pickles et al. demonstrated that an Ad5 vector failed to efficiently transduce MDCK cells overexpressing GPI-linked CAR following apical application, an effect that was reversible with removal of sialic residues by neuraminidase (52). In contrast, Walters et al. demonstrated that luminal application of an Ad2 vector efficiently transduced HAE cells overexpressing GPI-linked CAR on the apical membrane and that neuraminidase had no effect on gene transfer efficiency (53). Because Ad2 and Ad5 target the same region of CAR, it is unlikely that this incongruity results from differences in vector serotype. Rather, it may reflect differences in the levels of expression of glycocalyeal components between the different culture systems.

B. Vector-specific Barriers to Luminal Airway Gene Transfer

Cellular factors in airway cells may also affect the ability of gene transfer vectors to bind, enter, and translocate to the nucleus, and to express their transgenes after entry. These factors are discussed by vector below.

1. Ad Vectors

Adenoviruses are double-stranded DNA viruses, of which human serotypes 2 and 5 (90% homology) provide the backbone for current Ad vectors (54–57). Wild-type adenoviruses have a 36-kbp genome consisting of a series of early genes that are responsible for virus replication, antigen presentation and surveillance, and a series of late genes that encode viral structural proteins (54–57). Several generations of Ad vectors have been developed based on Ad serotypes 2 (Ad2) and 5 (Ad5). These generations are based on deletions of different regions of the Ad genome to improve safety.

In general, deletions of the early region one (E1) gene to make the vectors replication defective (58), with or without deletion of the E3 gene are characteristic of first-generation vectors. Additional deletions of the E2a region to form temperature-sensitive mutant viruses that may bring an additional safety feature to the vector (59–62), or alternatively, deletions in most [except for open reading frame six (ORF6)] or all the E4 region in an attempt to limit late viral gene expression (63–65), are characteristic features of second-generation vectors. Third-generation Ad vectors have had all their viral genes deleted, retaining only a small packaging signal and the inverted terminal repeats (66–72). These vectors can accept insert cDNAs or even genomic DNA in excess of 30 kb (56), but often require a stuffer sequence (70,72) for adequate packaging of vector constructs containing smaller cDNAs. Because these completely deleted vectors require coinfection of producer cells with an Ad helper virus for production of viral structural proteins, they are often referred to as gutless, high-capacity, or helper-dependent (HD) Ad vectors. Although the Ad helper virus may contaminate vector production stocks, the advent of Cre-lox recombination techniques has led to a reduction in the amount of Ad helper contamination to less than 0.5% to 1.0%.

Ad binds to a high-affinity CAR (73) on the cell surface and is internalized through an $\alpha_v\beta_{3/5}$ integrin-mediated vesicular (endocytic) process (74). As a result of endosomolytic properties mediated by the Ad penton base, Ad escapes the endosome and efficiently translocates to the nucleus, where it exists as an episome (extrachromosomal DNA)-mediating expression of therapeutic genes (cDNAs). Because Ad vectors do not

integrate at high frequencies, transient expression occurs so repetitive administration will be required for CF gene therapy.

Clinical trials and preclinical studies have established that luminal Ad gene transfer to airways is inefficient. Grubb et al. demonstrated that luminal application of an Ad-*lacZ* vector efficiently transduced basal cells, the predominant cell type at the site of mechanical injury in human and mouse tracheal explants, whereas uninjured lumen-facing columnar cells were resistant to gene transfer (75). This observation was confirmed in model systems of WD rat and human airway epithelia and extended to human intrapulmonary (bronchial) airways (76). Parallel experiments in excised human airway specimens demonstrated preferential transduction of undifferentiated regenerating or wound repairing cells by Ad vectors, as compared with WD pseudostratified columnar epithelia (77).

The causes of inefficient Ad gene transfer to WD airway epithelial cells following luminal application were delineated in subsequent studies. An early observation was that $\alpha_v\beta_{3/5}$ integrins were localized to the basolateral membrane, rather than the apical membrane, of columnar cells limiting vector entry (78). Subsequent experiments with radio- and fluorescent-labeled Ad vectors revealed evidence for decreased binding to the apical membrane of these polarized airway epithelial cells as compared with poorly differentiated airway cells, and a low rate of vector internalization out of proportion to the reduction in binding (76,79,80). Preferential transduction of polarized WD airway epithelia following basolateral application of vector as compared with the apical application was consistent with the binding and uptake studies (76,79,80). Immunofluorescent antibody studies ultimately localized CAR to the basolateral membrane (52).

Although the inefficiency of Ad-mediated gene transfer could be partially overcome by increasing the duration of Ad vector incubation with the epithelium (81,82), the markedly reduced rate of endocytic uptake in WD airway epithelia as compared with poorly differentiated airway epithelia (83) suggested that minimal enhancement of gene transfer would result from increasing nonspecific binding of Ad vectors to WD airway epithelia. Thus, vector-specific barriers to Ad-mediated gene transfer present on the apical membrane of WD airways lead to decreased uptake and decreased entry of vector.

2. AAV Vectors

Initially, AAV vectors were derived from the naturally defective and nonpathogenic wild-type human parvoviruses, AAV-2 and AAV-3 (84–88). The small genome (~4.7 kbp) of wild-type AAV-2 consists of the following: (1) inverted terminal repeats at the 5′ and 3′ ends of the molecule (84–87), which play a role in replication and are important for integration into the host cell genome; and (2) the viral genes *rep* and *cap*, which mediate viral replication and nucleocapsid formation. Deletion of *rep* and *cap* creates an AAV vector with an insert size of ~4.5 kb, which is at the upper size limit for insertion of full-length wild-type CFTR (coding region of ~4.5 kb) driven by an exogenous promoter. Thus, the small insert size of AAV vectors may serve as a barrier to gene transfer of human CFTR.

Poor binding and entry across the apical membrance of WD airway epithelia following luminal application of AAV vectors may also be limiting for CFTR gene transfer. A membrane-associated heparan sulfate proteoglycan has been identified as a receptor for AAV-2 (89) and fibroblast growth factor-1 (FGF-1) and $\alpha_v\beta_5$ integrins act as coreceptors for AAV-2 entry (90,91). Heparan sulfate proteoglycans and the coreceptors have been localized predominantly to the basolateral surface of WD HAE cells (45), which correlates with preferential transduction of these cells when vector is applied to the basolateral membrane relative to the apical membrane. However, binding of radiolabeled AAV-2 to the apical membrane of WD HAE cells was only reduced 4- to 7-fold reduction as compared with the basal membrane, which was incongruent with the 200-fold greater gene transfer efficiency detected following basal application. Thus, the existence of nonfunctional apical membrane receptors for AAV-2 in airway epithelia has been postulated (45,92).

Another barrier to transduction is inefficient conversion of single-stranded AAV genomes (ssDNA) into double-stranded DNA (dsDNA), a requirement for transgene expression (93,94). This limitation delays the onset of transgene expression, which can be overcome by waiting long enough for maximal transgene expression to occur (~4 weeks) or by the use of DNA damaging agents, topoisomerase inhibitors, and Ad early gene products (95–97). Thus, barriers to AAV-mediated transduction of CFTR into CF airways include small insert size, decreased binding, and uptake of vector due to absent or decreased functional receptor expression on the apical membrane, and inefficient single-strand to double-strand conversion of AAV genomes.

3. Retroviral Vectors

Retroviruses are members of a large group of viruses that infect vertebrates known as the Retroviridae (98,99). Several members of the mammalian C-type genus, which includes a variety of oncogenic retroviruses, and the lentivirus genus, which is associated with slowly progressive immunodeficiency states, have been developed into gene transfer vectors. Commonly used C-type retroviral vectors are based on murine leukemia virus (MLV). Lentivirus vectors derived from the human immunodeficiency viruses (HIV), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV) have each been developed as gene transfer vectors for cystic fibrosis.

Simple C-type retroviruses are RNA viruses whose genomes consist of 2 viral long terminal repeats (LTRs) that are important for cellular integration, but also contain promoter elements, a packaging signal, and a series of structural genes, *gag*, *pol*, and *env*. These structural genes encode the capsid protein, reverse transcriptase, protease, an integrase, and the envelope glycoprotein. Deletion of *gag*, *pol*, and *env* creates room for insertion of therapeutic cDNAs (genes) into the retroviral genome, forming a replication-defective retroviral vector (100). Production of the vector is performed by supplying

the deleted functions in *trans* with stable cell lines overexpressing helper proteins or by transient transfection.

Lentiviruses have a more complex genome than simple retroviruses that encode a variety of regulatory accessory proteins and pathogenesis factors that are not present in the genomes of simple retroviruses (101–104). Genes encoding proteins that use the cellular nuclear import machinery to target the preintegration complex to the nucleus are also encoded within this complex genome. Deletions in these accessory and pathogenesis factor genes have enabled the development of lentiviral vectors for gene transfer (101–104). Exogenous (internal) promoters have also been included within the sequences of the inserted gene cassette because transcription from the viral LTR may constitute a safety hazard. Deletions in the LTR to prevent transcription (self-inactivating or SIN vectors) have been introduced as a safety feature of retroviral vectors based on MLV and HIV (105,106).

The envelope glycoproteins of wild-type retroviruses and lentiviruses bind to cell surface receptors to facilitate entry of the virus into the cytoplasm where the viral RNA is reverse transcribed to form a cDNA, the provirus. This provirus is translocated to the nucleus where it integrates into the host cell chromosomes and through the normal process of DNA transcription, and encodes new viral proteins and new viral RNA, which are assembled at the cell surface into new viral particles. Replication-defective retroviral and lentiviral vectors infect cells by similar mechanisms, but unlike wild-type viruses, the integrated provirus from these vectors encodes the therapeutic gene and viral particles are not produced.

The lack of cell proliferation in WD airway epithelia in vivo (107) and low titers have traditionally served as major barriers to application of the C-type retroviral vectors derived from MLV to in vivo airway gene transfer efforts. The development of HIV, EIAV, and FIV vectors (101,102,104, 106,108,109), which can transduce nondividing airway cells, may overcome the requirement for cell proliferation by oncogenic retroviruses (110). Advances in retroviral production techniques and pseudotyping of vectors to create stable envelopes that permit concentration of vector stocks, may also soon overcome the limitations of titer (111). However, titers of retroviruses remain ~1 to 2 logs lower than that of Ad vectors or AAV vectors.

Apical membrane barriers to efficient transduction of WD airway cells are also limiting for retroviruses. Wang et al. demonstrated efficient transduction of polarized WD airway cells stimulated to proliferate with keratinocyte growth factor (KGF) when amphotropic-enveloped MLV vectors were applied to basolateral surface as compared with minimal to no gene transfer when vector was applied to the apical surface (112). The data are consistent with localization of the amphotropic receptor (RAM-1 or Pit-2) to the basolateral surface of polarized WD airway cells. Western blot data from this study suggested that receptor levels were extremely low in the absence of KGF. In vivo studies have confirmed low levels of expression of RAM-1 or Pit-2 in murine lung (113). However, Wang and colleagues have suggested that nonfunctional amphotropic receptors may be present on the apical membrane (114).

Similar findings have been observed with retroviral vectors bearing different envelopes. Wild-type vesicular stomatitis virus (VSV) preferentially infects polarized MDCK cells across the basolateral membrane (115). MLV and lentiviral vectors derived from HIV, EIAV, and FIV that have been pseudotyped with the envelope glycoprotein (G) of VSV also preferentially transduce polarized MDCK cells from the basolateral surface. In vitro and in vivo studies of transduction have confirmed this notion in MDCK cells, polarized WD HAE cells, and murine, rat, and rabbit tracheas in vivo (108,109,112,116–118). Thus, the lack of apical membrane receptors for retroviral or lentiviral vectors on the apical membrane of airway epithelia remains a significant barrier.

4. Nonviral Vectors

Cationic liposomes and naked plasmid DNA have been the principal nonviral vectors evaluated in CF clinical trials. Cationic liposomes are composed of cationic lipids mixed in varying molar ratios with cholesterol and dioleylphoshphatidylethanolamine (DOPE), a neutral phospholipid (119,120). A variety of cationic lipids have been used for gene transfer, including N[1-(2,3-dioleoxy)propyl] N,N,N trimethylammonium (DOTMA), 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide (DMRIE), or 3β[N-N′,N′-dimethylamino ethane-carbamoyl] cholesterol (DC-Chol), N[1-(2,3-dioleoxy)propyl] N,N,N trimethylammonium methyl sulfate (DOTAP), EDMPC cholesterol, and N^4-sperminine cholesteryl carbamate (GL-67). Cationic liposomes bind to negatively charged plasmid DNA to form DNA–liposome complexes (lipoplexes). These lipoplexes enter cells primarily by endocytosis (121,122), although the mechanism and specificity of binding to the cell surface has not been clearly delineated. Cationic liposomes do not integrate into the host cell genome, so that expression may be lost with cell division. Thus, repetitive administration of lipoplexes will be required for CF gene therapy.

Naked or plasmid DNA has also been shown to mediate gene transfer to lung epithelia in vitro and in vivo. In a clinical trial of lipid GL-67 in the nasal epithelium of cystic fibrosis patients, plasmid DNA alone was as effective as GL-67/CFTR plasmid DNA lipoplexes, although the restoration of chloride secretion was small in both cases (24).

Plasmid DNA can be linked to a polymer (e.g., poly-L-lysine), and a receptor ligand to form a DNA–ligand–polymer complex (polyplex). This complex may then bind to a specific cell surface receptor and enter the cell by receptor-mediated endocytosis. Polyplexes based on poly-L-lysine that use a Fab fragment of IgG against human secretory component have been shown to deliver reporter genes to the airway epithelia in vitro and in vivo following intravenous administration (123,124). These polyplexes appeared to preferentially target the larger cartilaginous airways where expression of the polymeric IgA receptor predominates, but were immunogenic.

Poly-L-lysine has also been linked to specific peptides that bind the serpin enzyme complex receptor (SEC-R) in an at-

tempt to increase luminal airway epithelial gene transfer efficiency (125). The poly-L-lysine polymer has also been modified to enhance gene transfer efficiency. Kollen et al. (126,127) have demonstrated that lactosylated poly-L-lysine can significantly enhance gene DNA transfer to cultured CF cells in vitro.

Two major barriers to nonviral gene transfer have been identified. The data have been primarily developed from studies of cationic lipids, but may also apply to polymers and naked DNA. Nuclear entry has been identified as the rate-limiting factor for efficient liposome-mediated gene transfer into cell lines resistant to transfection (128), whereas gene transfer to WD airway epithelial cells may be limited by both inefficient nuclear entry and failure of DNA–liposome complexes to enter the cell (83). In rat and human airway cells grown as islands on permeable collagen substrates with poorly differentiated cells forming the edges of the islands and polarized (WD) cells forming the central portions, loss of phagocytic entry mechanisms, decreased cell surface binding, and decreased uptake of lipoplexes into differentiated airway epithelial cells (central cells) as compared with poorly differentiated cells (edge cells) were detected as the reasons for inefficient transduction. These observations were confirmed in a parallel study that demonstrated decreased amounts of cell-associated lipoplexes in differentiated airway epithelia as compared with poorly differentiated epithelia (129). Because liposome-mediated gene transfer into proliferating cells was enhanced relative to quiescent cells, the possibility of enhanced nuclear transport of DNA during mitosis due to breakdown of the nuclear envelope was suggested. Thus, barriers to efficient transduction of WD airway cells by nonviral vectors include decreased binding and uptake of lipoplexes, as well as poor nuclear translocation of DNA. Polymer-based vector systems that target specific receptors on cell surfaces may overcome poor binding and uptake of nonviral vectors, but poor nuclear translocation with trafficking of significant portions of vector to lysosomal compartments for degradation remains a concern.

IV. OVERCOMING BARRIERS TO AIRWAY GENE TRANSFER

In the preceding section of this chapter, the apical membrane of WD airway cells has been established as a formidable barrier to efficient transduction by all the current gene transfer vectors. Intravenous approaches might overcome many of the apical membrane barriers (123,124), but the multiple barriers that must be crossed (e.g. endothelium, endothelial basement membrane, interstitium, epithelial basement membrane), combined with the increased possibility of systemic toxicity inherent in blood delivery, have made luminal delivery of vectors more attractive. Current strategies have not fully addressed the problem with luminal contents (i.e., neutrophils, bacteria, excess DNA, inflammation), focusing instead on improving apical membrane binding and entry. The hope is that pretreatment with a_1AT, rDNase, or antibiotic therapy will limit the effects of luminal contents on gene transfer.

Two major approaches have been proposed for overcoming barriers to airway epithelial gene transfer following luminal application of vector. One approach focuses on modification of the host airway to enhance gene transfer and the other modifies vectors to target receptors expressed on the apical membrane of airways in vivo that have the capacity to internalize. These approaches are discussed in more detail below.

A. Host Modification

1. Modulating Paracellular Permeability

The application of methods modulating paracellular permeability to enhance gene transfer has been well established. Inhalation of the oxidant gas sulfur dioxide promotes denuding of the surface epithelium in a dose-dependent manner, while increasing paracellular permeability in less severely injured regions (116,117,130). This oxidant model was used to stimulate epithelial cell proliferation and enable relatively efficient gene transfer to the airways of mice by VSV-G pseudotyped retroviral and lentiviral vectors (116,117). The surface active detergent polidocanol has also been shown to increase permeability of polidocanol-treated murine airways, but not that of control animals without inducing frank morphologic injury (42). Pretreatment of nasal airways with polidocanol-enhanced gene transfer mediated by an Ad-*lacZ* vector and facilitated partial correction of the Cl^- transport defect in the nasal epithelium of CF mice following a single dose of vector. The single dose of Ad-CFTR vector used following pretreatment with the surface agent polidocanol generated the same degree of CFTR correction previously reported by Grubb (75) in which 4 doses of an Ad vector were required to generate a 40% to 50% correction of Cl^- transport.

Agents that more specifically alter paracellular permeability of airways to enhance gene transfer have also been tested. Retroviral gene transfer mediated by an amphotropic enveloped vector applied to the apical membrane of WD airway epithelia was enhanced following pretreatment with the calcium chelator ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and hypotonic solutions (112). A 7- to 10-fold increase in transduction of WD primary HAE cell cultures by an AAV-GFP vector and transient permeabilization with EGTA/hypotonic solution has also been reported (45). EGTA/hypotonic solution treatment has enabled investigators to correct the Cl^- transport defect in WD CF HAE cells stimulated to proliferate with keratinocyte growth factor (KGF) when vector was applied to the luminal surface (112) and has enhanced FIV-based lentiviral gene transfer to rabbit airways in vivo (109), Ad gene transfer in vitro, and Ad gene transfer to murine airways in vivo (131). Chu et al. demonstrated that high concentrations of EGTA (0.1–0.4 M) were optimal for enhancement of Ad gene transfer to nasal and lower airway epithelia of mice in vivo (132). Medium chain fatty acids have also been shown to enhance airway gene transfer in vitro (133) and in vivo (133,134). Recently, lysophosphatidylcholine has been shown to enhance

HIV vector-mediated reporter gene transfer to murine nasal epithelia and to partially correct the the CF chloride permeability defect in the nasal epithelia of CF mice in vivo (135). These studies have established the feasibility of transient permeabilization of the paracellular path to enhance airway gene transfer in vivo mediated by vectors that bind to receptors that are localized on the basolateral membrane.

2. Enhancing Endosomal Processing

Duan et al. have suggested that receptors for AAV-2 may exist on the apical membrane of polarized WD HAE cells that can bind and mediate AAV-2 entry, but fail to mediate transgene expression (45,92). The rationale for this hypothesis was the observation that rAAV-2 transduction of WD HAE was 200-fold more efficient following basolateral application of 10^4 rAAV particles/cell as compared with apical application of an equal number of particles (45). This observation was incongruent with binding studies, which demonstrated only a 4- to 7-fold difference in binding between the apical or basolateral membranes. To further evaluate this observation, the investigators explored differences in the molecular state of rAAV 50 days after apical or basolateral transduction by Southern blot analysis of Hirt DNA (45). Following apical application of vector, rAAV genomes were detected consistent with cellular entry, but remained as ssDNA. In contrast, rAAV genomes that had been converted to dsDNA forms were detected after basolateral application of vector. DNA damaging agents did not increase gene transfer following apical infection, but the proteasome inhibitors z-LLL and LLnL, which prevent ubiquitination of molecules targeted for degradation, increased gene transfer efficiency to levels that were one-eighth of those observed following basolateral transduction. These data are consistent with a lumen-specific block to AAV endosomal processing and trafficking. Agents inhibiting proteasomal degradation also enhanced luminal rAAV-2-mediated airway gene transfer in vivo. These findings have not yet been verified by others in airways, although barriers to endosomal processing of AAV-2 have been identified in undifferentiated cell lines (136,137).

B. Vector Modification

Modifying the gene transfer vector is an alternative and perhaps, more popular approach to enhancing airway gene transfer efficiency. Retargeting the vector to receptors that are endogenously expressed on the apical membrane of WD HAE cells is the goal of this approach. The concept of targeting gene transfer vectors to alternative (non-wild-type) receptors is well established in the literature for adenoviral, retroviral, and nonviral vectors (138–149).

1. Ad Vectors

Methods for retargeting of Ad vectors have included the following: (1) genetic engineering of peptide ligand sequences or single-chain antibody fragments (scFv) into the fiber knob domain of Ad (139,148); (2) the adenobody approach, which is based on the use of scFv-fusion proteins with specificities for an Ad epitope while bearing a ligand-binding domain for a specific cell surface receptor (146); and (3) the use of bispecific antibodies composed of 2 antibodies—one directed against the Ad vector and the other against the specific cell surface receptor—that have been cross-linked (147,150).

A major concern for airway gene transfer has been which receptors are expressed on the apical membrane and whether the levels of expression are sufficient to promote successful gene transfer. $P2Y_2$-R, a 7 transmembrane purinoceptor that normally mediates acute airway epithelial cell responses to the luminal environment, the SEC-R, and the urokinase plasminogen activator receptor (uPA-R) have been proposed as potential targets on the apical membrane (125,151–154). In preliminary studies, bispecific monoclonal antibody-Ad vector complexes directed toward epitope-tagged external domains of $P2Y_2$-R can promote enhanced gene transfer efficiency to polarized cells (155). Targeted transduction of wild-type $P2Y_2$–R expressing cell lines mediated by vector conjugates composed of Ad complexed to the chemically modified ligand biotin-UTP has also been demonstrated (156). A uPA-polyethylene glycol (PEG)-coated Ad vector has also been shown to enhance Ad gene transfer to polarized airway epithelia (153,157).

Chimeric Ad vectors that target apical membrane of airway cells have also been developed. Zabner et al. screened 12 adenoviral serotypes from Ad subgroups A–F for their ability to bind and infect the apical surface of polarized WD HAE cells in culture (158). Wild-type Ad17 bound to WD HAE cells more efficiently than wild-type Ad2. Chimeric Ad2 vectors with Ad17 fiber were subsequently generated that transduced WD HAE cells ~100-fold more efficiently than the original Ad2 vector. These data suggested the feasibility of generating chimeric Ad vectors that more efficiently transduce HAE cells following luminal application.

2. AAV Vectors

Efforts to improve apical membrane targeting of AAV vectors have focused on identifying wild-type AAV vectors of other serotypes. These alternative serotypes have been developed into new gene transfer vectors or alternatively, their cap genes have served as pseudotypes for AAV-2 vectors. Six serotypes of AAV vectors have been cloned (159–161).

AAV-2 vectors pseudotyped with an AAV-6 capsid generated by transient transfection techniques have been developed (162). These AAV-6 pseudotypes bound heparin columns weakly as compared with AAV-2 and were not inhibited by soluble heparin sulfate, but luminally applied vector-transduced polarized primary CF HAE cells up to 100-fold more efficiently than AAV-2. AAV-6 pseudotypes also transduced a high percentage (up to 80%) of cells in some mouse airways in vivo that was 100- to 1000-fold higher than detected following AAV-2 gene transfer. Of note, AAV-6-pseudotyped AAV-2 vectors were produced in much higher titers than AAV-6 vectors, suggesting that pseudotyping may offer advantages for vector production. No studies of AAV-6 or AAV-6 pseudotyped (AAV capsid) vectors containing CFTR transgenes have been reported to date.

The molecular characteristics of AAV-4 and AAV-5 in comparison to AAV-2 have been reported (163). Significantly more AAV-4 and AAV-5 bound to the apical membrane of WD HAE cells than AAV-2, but only AAV-5 efficiently transduced airway cells following apical application. AAV-5 transduction was not inhibited by pretreatment with soluble heparin consistent with the use of a different receptor for binding and uptake of vector from AAV-2. AAV-5 vector-transduced lungs exhibited a 5-fold increase in the number of transduced cells/microscope field for airways and 20- to 30-fold increase for alveoli as compared with AAV-2-transduced lungs. Current data suggest that 2,3-linked sialic acid residues may serve as the receptor for AAV-5 (164). Thus, AAV-5 and AAV-6 may be candidates for enhancing AAV gene transfer following luminal delivery. However, confirmatory studies of gene transfer efficiency, CFTR transduction, and safety will be required.

The restriction on insert size that limits efficient packaging of wild-type CFTR with a suitable promoter has been an additional barrier to AAV-mediated CFTR gene transfer. Expression from AAV-CFTR vectors in clinical trials has been driven by endogenous promoter elements within the viral terminal repeats due to limits on insert size (19,20,165). Because AAV vectors undergo intermolecular circular concatermerization and recombination, a trans-splicing strategy has been proposed for full-length cDNAs in excess of the packaging size limits (166). In this strategy, rAAV vectors encoding either the 5′ or 3′ portions of a therapeutic transgene with splice donor sites intact would be delivered into the same cell where circular concatamers arising from intermolecular recombination permit functional trans-splicing of the component portions of the molecule into full-length therapeutic cDNAs (166). This strategy has been successfully applied to the human erythropoietin (Epo) gene in vitro and to animal models of renal failure-induced anemia (166). A similar strategy has been proposed to restore CFTR chloride channel function to CF epithelia, but the feasibility of this approach will depend on efficient delivery of the vector encoding each half of CFTR, or alternatively, a CFTR transgene in one vector and a promoter with a superenhancer in another to the same cell with a high frequency of trans-splicing (167).

An alternative methodology for introducing larger transgenes into WD airway cells in vitro and in vivo has recently been published (168). Halbert et al. have delivered 2 AAV vectors containing overlapping homologous fragments (AAV rec vectors) of the human placental alkaline phosphatase gene (AP) gene into airway cells in vitro and in vivo. Theoretically, homologous recombination between the fragments from the 2 different vectors would result in expression of the transgene. Delivery of the AAV rec vectors in vitro resulted in inefficient recombination and expression in vitro. However, AAV6 capsid AAV rec vectors transduced murine airways in vivo at nearly the same efficiency (~10%) as an intact AAV6 capsid-AP vector (168). These data suggested that application of alternative AAV pseudotypes combined with transplicing or AAV rec vector technology may overcome both the apical membrane and insert size limitations common to AAV-2 vectors.

3. Retroviral Vectors

Approaches that overcome in vivo barriers to retroviral transduction must address issues of titer, cell proliferation, and receptor localization. Retroviral vector titers have traditionally been in the range of 10^5 to 10^6 infectious units (IU)/mL, with further increases in titer limited in part by the stability of the envelope protein. Envelope proteins from a variety of viruses can be incorporated into the viral membrane of retroviral vectors, a process known as pseudotyping, which is common to most retroviruses including lentiviruses.

VSV-G pseudotyped MLV and lentiviral vectors have been developed that can be concentrated to high titer by ultracentrifugation without significant loss of infectivity (101–104, 108,109,111,116,169). Following concentration, retroviral titers approach titers within a log of AAV and Ad vectors and high titer VSV-G pseudotyped MLV and lentiviral vectors have been shown to correct the Cl^- permeability defect in poorly differentiated dividing (subconfluent), and WD primary CF HAE cells without selection (116,118). The toxicity of VSV-G envelope protein and some lentiviral accessory proteins initially limited the ability to develop stable packaging cells lines for vector production, but regulated expression of helper and envelope components with tetracycline-inducible promoters has permitted the development of stable VSV-G pseudotyped MLV and lentiviral vector producer lines (170–172).

Oncogenic retroviral vectors based on MLV require cell proliferation for nuclear entry, integration, and subsequent transgene expression. Two models have been developed to stimulate epithelial cell proliferation in vivo: (1) oxidant gas injury models, and (2) growth factors (109,112,113,116–118). Although oxidant gas injury and growth factors can lead to successful in vivo airway gene transfer with MLV-based vectors, the ability of lentiviral vectors to transduce a variety of nondividing cell types in vitro and in vivo has largely overcome the limitation of cell proliferation typical of oncogenic retroviruses (101–104,106,108,109,117,169). Johnson et al. evaluated whether HIV (VSV-G) vectors could transduce nondividing airway epithelial cells (117). An amphotropic enveloped vector [MLV (ampho)], HIV (VSV-G) vector, and MLV (VSV-G) vector each efficiently transduced growing or dividing CF tracheal epithelia cells (CFT1), whereas only the HIV (VSV-G) vector efficiently transduced aphidicolin-treated (growth-arrested) airway epithelial cells.

The envelope proteins of lentiviral vectors bind to cell surface receptors to facilitate entry with subsequent reverse transcription, nuclear import, and integration into the host chromosome culminating in gene expression. To evaluate the polarity of lentiviral transduction, VSV-G pseudotyped vectors have been applied to either the apical or the basolateral surfaces of polarized WD HAE cells. Thirty-fold greater transduction efficiency was detected in vitro when HIV (VSV-G) vectors were applied to the basolateral surface as compared with apical application of vector (173). Goldman and col-

leagues demonstrated that luminally applied HIV (VSV-G) vectors failed to transduce WD primary HAE cells in bronchial xenografts (173). Similar data have evolved for EIAV pseudotyped with VSV-G (J.C. Olsen, unpublished observations 1999), and for amphotropic and VSV-G enveloped FIV vectors (109,131). These data suggest that, although lentiviral vectors can transduce nondividing cells, the receptors for uptake and entry of amphotropic and VSV-G pseudotyped MLV and lentiviral vectors are predominantly localized to the basolateral membrane of polarized epithelial cells.

Two strategies have been proposed to overcome the lack of apical membrane receptor expression: (1) host modification with injury models and agents that increase paracellular permeability to increase vector access to basolateral membrane receptors and basal cells (173), and (2) targeting the apical membrane of polarized airway epithelia by pseudotyping lentiviruses with envelope proteins from other viruses that bind and enter across the apical membrane. Host modification has been discussed above. Targeting lentiviral vectors to receptors on the apical membrane to increase airway gene transfer are discussed here.

Retroviral targeting is typically approached by generating pseudotypes from envelope proteins of other viruses that target specific cell types. To identify potential pseudotypes tropic for the apical membrane of WD HAE cells, investigators have screened enveloped viruses for their ability to infect these cells following luminal application. Efforts have focused not only on common respiratory viruses, but have also included nonrespiratory viruses. Respiratory viruses considered as candidates for pseudotyping include respiratory syncytial virus (RSV), human corona virus, and influenza virus. Wild-type and replication-competent recombinant RSV infect or enter cells across the apical membrane of WD HAE cells (174,175). Human corona virus 229E (HcoV229E) has also been shown to bind and enter across the apical membrane of WD HAE cells (176). However, no successful lentiviral pseudotypes derived from the Env proteins of RSV and HcoV229E have been reported.

Preferential infection of the apical membrane of WD HAE cells by influenza A virus subtype H2N2/Japan/305/57 has been reported, whereas apical infection of HAE cells with subtypes H1N1 and H3N2 was inefficient (177). Apical membrane binding of the H2N2 subtype was specific for sialic acid α2,3-gal residues, suggesting that these sialic acid residues serve as apical membrane receptors for targeting of lentiviral pseudotypes. Morse et al. have shown in preliminary studies that coexpression of influenza M2 and neuraminic acid (NA) proteins in producer cells enhances titers of influenza hemagglutinin pseudotyped EIAV vectors (178). Moreover, these HA/M2/NA chimeric pseudotyped vectors transduced polarized WD HAE cells following luminal application.

The Env proteins of other viruses have also been considered. In a preliminary study, FIV-based vectors were pseudotyped with amphotropic, xenotropic, VSV-G, RD-114, 10A1, ecotropic, GALV, Marburg, and Ebola envelope glycoproteins by transient transfection techniques. Only FIV vectors pseudotyped with the Marburg virus envelope glycoprotein efficiently transduced WD HAE cells following luminal application (179).

The potential of the family of viruses known as the Filoviridae to target the apical membrane of airway epithelia (180) has been explored by Kobinger and colleagues. Filoviridae are enveloped nonsegmented negative sense RNA viruses, which include the Ebola and Marburg viruses among its members. To identify viral envelopes capable of mediating apical transduction of polarized air–liquid interface cultures, HIV vectors were pseudotyped with MLV, influenza-hemagglutinin, RSV F and G proteins, Mokola, Ebola Reston (Ebo-R), or Ebola Zaire (EboZ) envelopes. EboZ, but not EboR or other pseudotypes, efficiently transduced polarized HAE cells following apical application with gene transfer in up to 70% of cells as compared with up to 40% of cells following basolateral transduction. EboZ-pseudotyped HIV vectors also transduced WD HAE cells in xenografts and in freshly excised human tracheal explants. Thus, lentiviral vectors may overcome the limitations of low rates of cellular proliferation and pseudotyping lentiviral vectors with specific envelope proteins may overcome barriers to titer and the lack of apical membrane receptors on polarized WD HAE cells.

4. Nonviral Vectors

Poor apical membrane binding, and entry and inefficient nuclear transport, must be overcome to improve the efficiency of nonviral gene transfer in human airways. Until recently, cationic lipids and naked DNA had been the only nonviral vectors evaluated in CF clinical trials. Newer nonviral gene transfer vectors have been developed based on polymers that may have advantages over cationic lipids and 1 protocol polymer-based human clinical trial for cystic fibrosis has been registered with Office of Biotechnology Activities (OBA), National Institutes of Health. Polymers are inherently more flexible because they can readily be linked to ligands preferentially targeting the apical membrane. The localization of the SEC-R to the apical membrane of WD HAE cells has led to the development of polymer complexes (polyplexes) of SEC-R targeted peptide ligand-poly-L-lysine-DNA (125,154). These polyplexes efficiently targeted the apical membranes of murine nasal epithelia in vivo and partially corrected the functional chloride permeability defect in CF mouse nasal epithelia (181). Lactosylated poly-L-lysine has also been suggested as a nonviral polymer that may promote binding to the apical membrane of airways (126,127,182). Although this vector has shown promise in vitro, the ability to mediate efficient transduction of polarized WD HAE cells in vitro or in vivo has not been reported.

Fusogenic peptides, weak bases (chloroquine), and glycerol, which presumably has an osmotic effect on intracellular vesicles, have been proposed as agents promoting endolysosomal escape in an attempt to overcome inefficient nuclear transport of nonviral vectors, (183). The utility of these agents has not been verified in WD HAE cells in vitro or in vivo. Polyethylenimine polymers may also promote endosomal release (184), but appear to lack specific targeting moieties. Recent studies have suggested that polyplexes of lactosylated

poly-L-lysine and SEC-R targeted poly-L-lysine with condensed DNA may be more efficiently translocated to the nucleus (183,185). Lactosylated poly-L-lysine-DNA complexes appeared to enter undifferentiated CF primary and immortalized cells by a receptor-mediated process. Confocal micrographs of labeled complexes demonstrated rapid perinuclear accumulation of lactosylated poly-L-lysine polyplexes with a small amount of nuclear entry. However, agents that inhibit lysosomal trafficking markedly increased nuclear delivery, suggesting that the majority of the lactosylated polyplexes trafficked to the endolysosomal pathyway (183).

In preliminary studies of human hepatoma cells (HuH7), fluorescently labeled SEC-R-targeted complexes, which enter the cell by receptor-mediated endocytosis, were delivered to the perinuclear region within 5 min of binding with some free DNA, but no poly-L-lysine appeared in the nucleus (185). In contrast to studies with lactosylated poly-L-lysine, chloroquine did not increase gene transfer mediated by SEC-R-targeted polyplexes, consistent with nuclear translocation with efficient escape from the endolysosomal pathway (185). These data regarding the targeting of these polyplexes have not been verified in polarized airway cells.

5. Novel Systems

The paramyxoviruses have shown promise for gene transfer (186,187). Sendai virus (SeV) is an enveloped single-stranded nonsegmented negative strand RNA virus that mediates cell attachment and entry via the envelope glycoproteins hemagglutinin-neuraminidase (HN) and fusion protein (F). The ability of the ribonucleoprotein (RNP) of this virus—and paramyxoviridae family members in general—to mediate transcription and replication independent of nuclear functions or a DNA phase permits infection of nondividing cells. Reverse genetics has permitted the development of replication-competent SeV vectors and recently a nontransmissable SeV vector has been developed (187). Replication-competent SeV vectors have been shown to mediate efficient reporter gene transfer to murine lung throughout the conducting airways with expression present in ciliated columnar, nonciliated secretory cells, and submucosal gland cells, but not basal cells (186). Similar results were obtained following delivery to ferret lung in vivo and to freshly excised human airways ex vivo. Importantly, only a modest inhibition (50%) of SeV-mediated gene transfer has been reported in the presence of mucus. Dose-dependent inflammatory responses, which were detected in third-generation and smaller airways, and the loss of SeV-mediated expression with cell division are potential limitations of this vector.

Respiratory syncytial virus and parainfluenza virus-3 are other members of the paramyxoviridae family of viruses that have been considered for new vector development. RSV mediates cell entry through its glycoproteins G and F (188). Like SeV, transcription and replication are cystoplasmic events for both RSV and PIV-3. In preliminary studies, a replication-competent RSV-GFP virus efficiently transduced WD HAE cells following apical, but not basolateral, application of vector (174). Similar findings have been noted in WD HAE cells for PIV-3 (189). RSV and PIV-3 will likely have similar limitations as SeV for vector development. However, SeV, RSV, and PIV-3 offer exciting promise for future human airway gene transfer efforts.

V. SUMMARY

The airway epithelium is a formidable target for gene transfer approaches due to its complex anatomy, large surface area, innate immune functions, and the presence of specific barriers that limit entry of foreign particles. The inflammatory response in CF airways only serves to fortify the barrier to airway gene transfer. Nevertheless, progress has been made and several approaches have evolved to address the luminal barriers to gene transfer. Efforts to improve the ability of vectors to target the apical membrane of airway epithelia in vivo and methods that modify the host to increase vector access to cell targets offer hope, but more basic research will be required to achieve successful gene therapy of CF lung disease.

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29

Gene Therapy for Neurological Diseases

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I. INTRODUCTION

Highly effective methodologies for therapeutic gene delivery to cells of the central nervous system (CNS) have borne from the exponential gains achieved in the area of molecular biology over recent decades. This capability has created promise for therapeutic intervention in neurodegenerative disorders. Presently, the most widely employed treatments for neurodegenerative diseases are based symptom alleviation through the use of pharmacological compounds, but these approaches offer little curative, or even neuroprotective properties. An ideal therapy for a neurodegenerative disorder would stabilize and/or repair afflicted cells and prevent other cells from succumbing to the pathophysiological process. Using gene transfer-based methodologies, it may be possible to create such therapies. The characteristics of presently available virus-based vectors are summarized in Table 1. Each vector platform exhibits a series of advantages and disadvantages that need to be assessed. However, prior to their implementation in the treatment of neurological disorders, a detailed understanding of the disease-specific mechanisms is crucial. These issues are addressed in greater detail below.

A. Therapeutic Transcriptional Unit

A thorough understanding of the molecular mechanisms underlying the neurodegenerative disease process is requisite for selection of a proposed therapeutic gene. In general, the therapeutic transcriptional unit should have 1 or more of the following properties: (1) neuroprotective, (2) neuroregenerative, (3) neurotrophic, or (4) antiapoptotic.

Equally important is choosing promoter(s) and regulatory element(s) to drive expression of the desired gene(s). The choice of these components will determine the specificity, duration, and regulation of transgene expression. Once the transcriptional unit has been designed, a suitable vector for delivering the DNA must be selected. The vector must be capable of delivering and expressing the desired genes in the appropriate cells to be targeted.

B. Vector Selection

Subsequent to selection of the gene therapeutic effector gene, whether it possesses a neuroaugmentative or neuroprotective mechanism of action, the appropriate gene delivery vector can be chosen. The following points must be considered for informed selection of an appropriate gene therapeutic vehicle for the treatment of neurodegenerative disorders: vector capacity, vector specificity, vector genome maintenance, vector-mediated transgene expression duration and levels, and vector safety profile. The length of the therapeutic transcription unit is sometimes used as a first criterion to limit vector choice. This category also includes a given vector's ability to harbor multiple transcription units, thereby potentially affording recapitulation of a complex biochemical pathway (i.e., dopamine biosynthesis in the case of Parkinson's disease). Potential applications for several presently available vectors are restricted by insertion size limitations and are sometimes excluded if multiple gene delivery is required for therapy. As demonstrated with adeno-associated virus (AAV) vectors, the coinjection of multiple vectors, where each expresses a different therapeutic gene, an approach to circumvent this issue (1).

Cell type specificity is also an important issue when developing a gene-based therapeutic intervention for neurodegenerative disorders. It would be most beneficial if the vector of choice could transduce and express in cell types that comprise the disease-affected neural pathway. Vector tropism can be

Table 1 Viral Vector Platforms Employed in CNS Gene Transfer

Viral vector	Payload capacity	Tropism	Duration expression	Other properties
Adenovirus	7–8 kb (Gen[a] 1/2); 30 kb (Gutless)	Glial/Neuronal	Months (Gen1/2); Year (Gutless)	Episomal; immunogenic (Gen 1/2); less immunogenic (gutless)
AAV	4.5 kb	Neuronal	Years	Episomal and integrative; minimal immune response
HSV recombinant	~10 kb	Neuronal> Glial	Months	Episomal; some cytotoxicity
HSV amplicon	~130 kb	Neuronal> Glial	Months to a year	Episomal; all viral genes deleted; little immune response
HSV/AAV hybrid amplicon	>20 kb	Neuronal	Months to a year	Episomal and integrative; all viral genes deleted
Lentivirus	8–10 kb	Neuronal	Years	Integrative

[a] Gen1/2, generation 1 and 2.

regulated through modulation of cellular receptor interactions by 1 or more of the following approaches: alteration of virus docking proteins, utilization of alternate serotypes, pseudotyping, and introduction of cellular receptors into the viral envelope. Once a vector is optimally targeted to the brain region of interest, therapeutic transgene expression can be restricted to selected cell populations via the utilization of cell type-specific promoters and/or transcriptional elements (2–6). Strict spatial control of transgene expression is important to ensure the correct cells will manufacture the gene product. This control, in turn, reduces the risk that ectopic transgene expression will occur and lead to untoward effects on adjacent neurological pathways.

Because many neurodegenerative disorders are protracted in duration, gene-base modalities will be required to impart therapeutic benefit for several decades of an individual's lifetime. To this end, a vector genome should be stably maintained within the transduced cell of the neural pathway for extended periods of time. Vector genome maintenance is, therefore, an important factor in selection of an appropriate gene therapy vehicle for such disorders. Vector genomes can exist episomally and/or as integrated forms within nuclei of transduced cells. The postmitotic property of CNS neuronal populations does not exclude the utilization of episomal vectors because genomes can be maintained without the fear of progressive loss due to mitosis. Integrating vectors circumvent this issue but their use augments safety concerns, including potential to transactivate nearby proto-oncogenes and to disrupt essential host genes via insertional mutagenesis. This fear has apparently become a reality as evidenced in the case of the children in Europe treated for X-linked severe combined immune deficiency disease (X-SCID) with a murine retroviral vector expressing the T cell growth factor, gammaC. Two of 10 enrolled infants developed similar leukemia-like illnesses, and the trial was halted to determine the genesis of the adverse event (7).

Another similar issue regarding vector selection relates to the desired levels and duration of gene product expression for treatment of neurodegenerative disorders. Depending the vector and transcriptional elements chosen, pharmacological or physiological levels of transgene expression can be achieved for short- or long-term periods of duration. As with other selection criteria, the decision of which level/duration of expression is preferred rests heavily on the underlying molecular mechanisms to be targeted and at which time during the disease course the intervention is to be implemented. Early interventions may require maintenance of long-term physiological levels of transgene expression (i.e., neuroprotective strategies) because the neuronal system is likely to be intact at this time. A vector/promoter combination that safely and stably maintains gene expression at nearly physiological levels in the CNS would serve as a potential candidate for such early treatment approaches. Treatment modalities that are implemented after presentation of clinical neurodegenerative disease symptoms may require long-term pharmacological levels of transgene product to restore neurological function to a brain region decimated by disease.

Safety is of utmost concern regarding the application of novel gene therapeutic strategies to the treatment of neurodegenerative disorders. Many presently available vectors trigger immunogenic and/or inflammatory responses when introduced into the CNS. These responses are known to arise from the humoral and/or cell-mediated arms of the immune system, and the magnitude differs depending which vector type is employed. For example, repeat administration of early generation viral vectors has been shown to lead to lower transgene expression and serious inflammation, likely the result of a primed immune system (8). Therefore, a vector that is stably maintained and that can express its encoded transgene for extended periods of time would likely prove to be a more favorable choice as a gene therapeutic vehicle for neurodegenerative disorders. Another aspect that is often overlooked regarding gene therapy safety is the role of transgene products in the elaboration of immune responses and toxicity. Transgene products that are of foreign origin, ectopically expressed, or

pharmacologically expressed possess the potential to induce cytotoxicity and/or immune responses. Research addressing these issues is imperative to elucidate the role of transgene products in the elaboration of these potentially harmful responses, and how such responses can be successfully circumvented. Utilization of regulation-competent transcriptional or posttranscriptional elements in delivery vectors to provide ''fine-tuning'' of therapeutic transgene expression levels is a way to minimize harmful clinical outcomes.

C. Vector Delivery

Once a suitable vector has been chosen for treating a particular neurological disease, a safe means for delivery must be established to provide optimal therapeutic benefit. With recent refinements of stereotactic surgical procedures, highly precise and reproducible delivery to specific regions of the brain are now performed [reviewed in (9)]. By placing the patient's head within a rigid frame and using 3-dimensional cartesian reference points, delivery of vectors or cells can be made to a defined space within the coordinate system. Gene therapeutic vectors can be introduced into the brain via direct or indirect means. Direct gene transfer involves either local or global delivery of a selected vector to the brain. Direct local delivery using stereotactic methods is highly suitable for treatment of certain neurological diseases due to the fairly circumscribed region that may be afflicted. Figure 1 illustrates the finely tuned and restricted delivery of an herpes simplex virus (HSV) amplicon vector expressing the β-galactosidase gene within the striatum of a mouse (10).

Other investigative groups have used more global approaches to CNS gene delivery that have broader applications for many neurological diseases where the volume of affected brain region is beyond the feasibility of conventional stereotactic methods. Convection-enhanced delivery (CED) has been developed to distribute homogeneous tissue concentrations of vectors over a large region of rodent or primate brain (11–14). This method has been used extensively in the development of gene therapeutic strategies for neurodegenerative disorders, but further research is required to determine if global distribution of vectors produces deleterious effects on neural pathway functioning. For example, global expression of vector-derived neurotrophic factors may induce uncontrolled neuritic sprouting, and subsequently, altered neuronal activity and physiology.

Indirect gene transfer, or ex vivo therapy, uses transplantation of genetically altered (vector-transduced) or unaltered cells capable of restoring functionality to a diseased region. Although ex vivo therapy includes the use of neural stem cells for repopulation of the denervated dopaminergic system and restoration of pathway function, this type of therapy is be discussed in this review. Vectors used to genetically modify transplanted cells typically express either secreted trophic factors for neuroprotection/neuroaugmentation of surrounding host tissues or cell-intrinsic survival factors for protection of

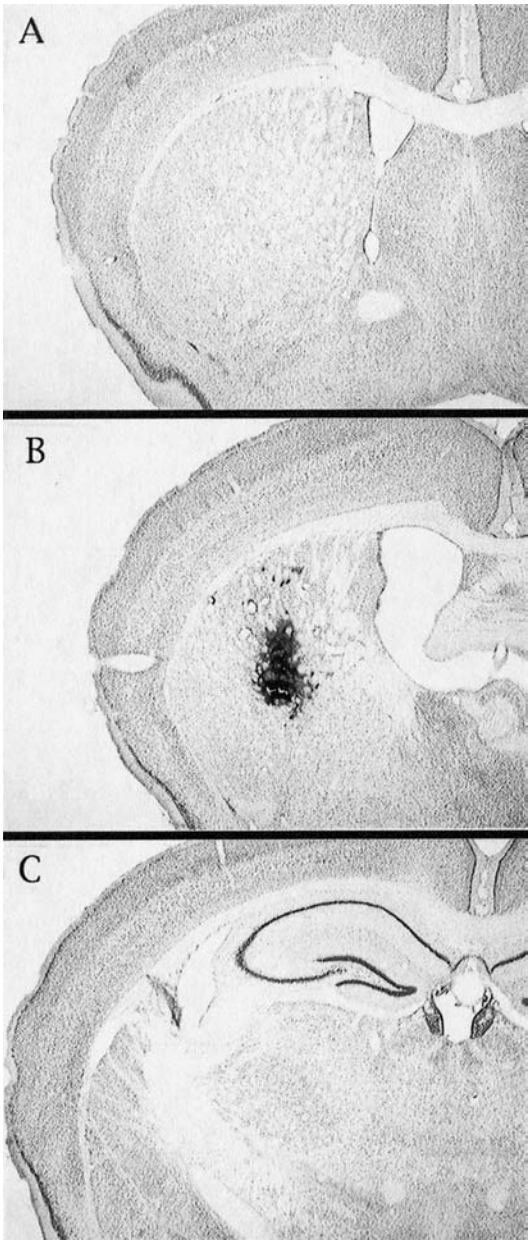

Figure 1 Stereotactic delivery of an HSV amplicon vector expressing β-galactosidase into the mouse striatum. Mice were injected with 1×10^5 transduction units of HSVlac using a microprocessor-controlled pump. Animals were sacrificed and perfused 4 days posttransduction and X-gal histochemistry was performed on 40-μm sections. Sections representative of the injection site (A), a site anterior of the injection (B), and a site posterior of the injection (C). All sections were counterstained with thionin and acquired at a magnification of 2.5×. The photomicrographs indicate that focal delivery of a viral vector can be achieved within the brain. (From Ref. 10, © 1998 Elsevier Science B.V.) See the color insert for a color version of this figure.

grafted cells from the stress of transplantation. Vectors delivered via ex vivo therapy must be able to integrate into the host cell genome because transplanted cell populations are expanded prior to grafting. Utilization of gene transfer technologies may allow for the expansion of the graft cell population or increased graft survival via expression of a growth or survival factor, thereby minimizing the amount of starting fetal tissue (15,16).

II. NEURODEGENERATIVE DISEASES AMENABLE TO GENE THERAPY

The following discussion illustrates the most common forms of neurodegenerative diseases (summarized in Table 2) and potential gene therapy approaches that have been employed based on current understandings of disease mechanisms.

A. Parkinson's Disease

Parkinson's disease (PD) is the second most common chronic neurodegenerative disease of humans. In 1995, the incidence of PD was estimated to be between 1:100 to 1:500 individuals (17,18). This incidence translates to approximately 1% of the population over the age of 65 (19–21). The disorder, initially described in 6 patients by Dr. James Parkinson, is typified clinically by symptoms including bradykinesia, resting tremor, rigidity, and gait abnormalities, followed by postural instability, dementia, and autonomic dysfunction. Pathologically, PD patients experience specific degeneration of dopaminergic neurons in the substantia nigra pars compacta as well as dopaminergic ventral tegmental area (VTA) neurons and noradrenergic neurons of the locus coeruleus. Furthermore, neuronal loss has been reported in other brain areas such as the cerebral cortex, anterior thalamus, hypothalamus, amygdala, and basal forebrain. In addition to neuronal loss, accumulation of proteinaceous cytoplasmic inclusions called Lewy bodies is a neuropathological hallmark of PD. The exact role of Lewy bodies in PD is unclear, but other neurodegenerative disorders also exhibit intracellular and extracellular protein aggregates. Understanding the molecular mechanisms underlying protein aggregates in neurodegenerative diseases may assist to illuminate a common target for gene therapy.

Although more than 180 years have passed since Parkinson's first description, the disease etiology is still largely unknown. Because the evolution of successful gene therapeutic strategies will rely heavily on a detailed understanding of the molecular and cellular processes governing the clinical presentations of PD, exploring the pathophysiology of PD is crucial. The following section summarizes possible mechanisms of PD and how methods in gene delivery and expression might be applied to interdict the pathogenic pathway(s) of this neurodegenerative disorder.

1. Mechanisms of Disease

PD exists as both a sporadic and familial disorder. Although the exact etiology of PD is unknown, the common pathway of both sporadic and familial PD is a loss of dopamine (DA) neurons. Importantly, this decline in DA neuron number below a critical threshold produces early symptomatic PD [reviewed in (22–24)]. Given that environmental factors such as pesticides, herbicides, and industrial chemicals have been

Table 2 Neurological Disorders Amenable to Gene Therapy

Disease	Age onset	Disease mechanism	Basis
Parkinson's	50–70s	Loss pigmented dopamine neurons in midbrain	*Genetic* (rare): α-synuclein, C-terminal ubiquitin hydrolase; parkin *Environmental*: pesticides, fungicides, neurotoxicants
Alzheimer's	50–80s	Aβ accumulation; tau hyperphosphorylation; synapse loss	*Genetic* (infrequent): APP, PS (susceptibility): ApoE4 *Environmental*: Viral infection/diet?
Lysosomal storage	Infancy	Enzyme deficiency leading to lysosomal protein accumulation and eventual peripheral and CNS degeneration	*Genetic*: lysosomal enzyme/transport gene deletion/mutation
Huntington's	20–50s	Loss of striatal medium spiny neurons	*Genetic*: Polyglutamine expansion in huntingtin gene locus
Stroke	Varies	Hypoxic insult leads to necrotic and apoptotic cell death	Potential genetic and environmental susceptibility interaction
Epilepsy	Childhood	Aberrant hypersynchronization of local or global neuronal networks	Unknown: potential early lifetime subclinical event
Motor neuron disorders	40–60s	Loss of spinal motor neurons	*Genetic*: missense mutations (ALS); gene deletions/aberrant splicing (SMA)

identified as potential risk factors for PD and genetic mutations have also been identified, it is likely that either alone or in combination, these triggers will produce a clinical syndrome similar to PD [reviewed in (24)]. Figure 2 outlines a common pathway for PD and suggests several targets for gene therapy without necessarily interdicting the initiating mechanism. In this ''common pathway'' model for PD, multiple triggering mechanisms such as genetic, toxicant, and environmental trigger, plus genetic vulnerability, converge on a shared common pathway to cell death. The first step along this pathway may encompass presynaptic injury and dysfunction followed by cellular compensation and metabolic stress. This preclinical injury and damage would result in DA deficiency and cell death. Gene therapy treatment opportunities would include (1) targeting specific triggering mechanisms, (2) targeting shared early pathways prior to presynaptic dopaminergic dysfunction, (3) targeting shared later pathways when dysfunction occurs, and (4) restoring DA biosynthesis in the denervated striatum. As stated above, PD is likely the result of a combination of environmental, toxin, and genetic triggering factors. Because of this, it is difficult to review the impact of each individually, but we attempt to briefly outline the potential impact of these factors on the etiology of PD and summarize their convergence on the pathophysiology of this disease.

2. Environmental Factors

Several findings support an etiologic role for exogenous factors in PD. The earliest observation was that a synthetic by-product of meperidine production produced a syndrome similar to PD. 1,2,3,6-Methyl-phenyl-tetrahydropyridine (MPTP) treatment of mice and monkeys has become a common method to achieve dopaminergic neuronal loss and an animal ''model'' of PD. The toxic compound MPTP is converted in glia to the pyridinium ion (MPP+) by monoaminoxidase type B (MAO-B) and subsequently taken up by dopamine neurons via the dopamine transporter. MPP+ is then actively transported to the mitochondria where it inhibits complex I, interfering with mitochondrial respiration and resulting in increased production of the superoxide anion. (25).

Recent reports lend biological plausibility to pesticide exposure as both an alternative model of nigrostriatal dopaminergic degeneration and a risk factor for PD (26–28). Greenamyre's group reported that chronic, systemic treatment of rats with the lipophilic pesticide, rotenone, resulted in highly selective nigrostriatal dopaminergic degeneration (27,29). This degeneration is associated with PD-like behavioral changes, including hypokinesia and rigidity. Neuropathologically, these animals accumulate fibrillar cytoplasmic inclusions that contain both ubiquitin and α-synuclein. This treatment represents an important alternate PD model because these inclusions resemble Lewy bodies, a hallmark of PD. Of equal importance is the suggestion from this model that mitochondrial dysfunction in dopaminergic neurons, in particular, complex I inhibition, plays an important role in the pathophysiology of Lewy body formation and some cases of idiopathic PD.

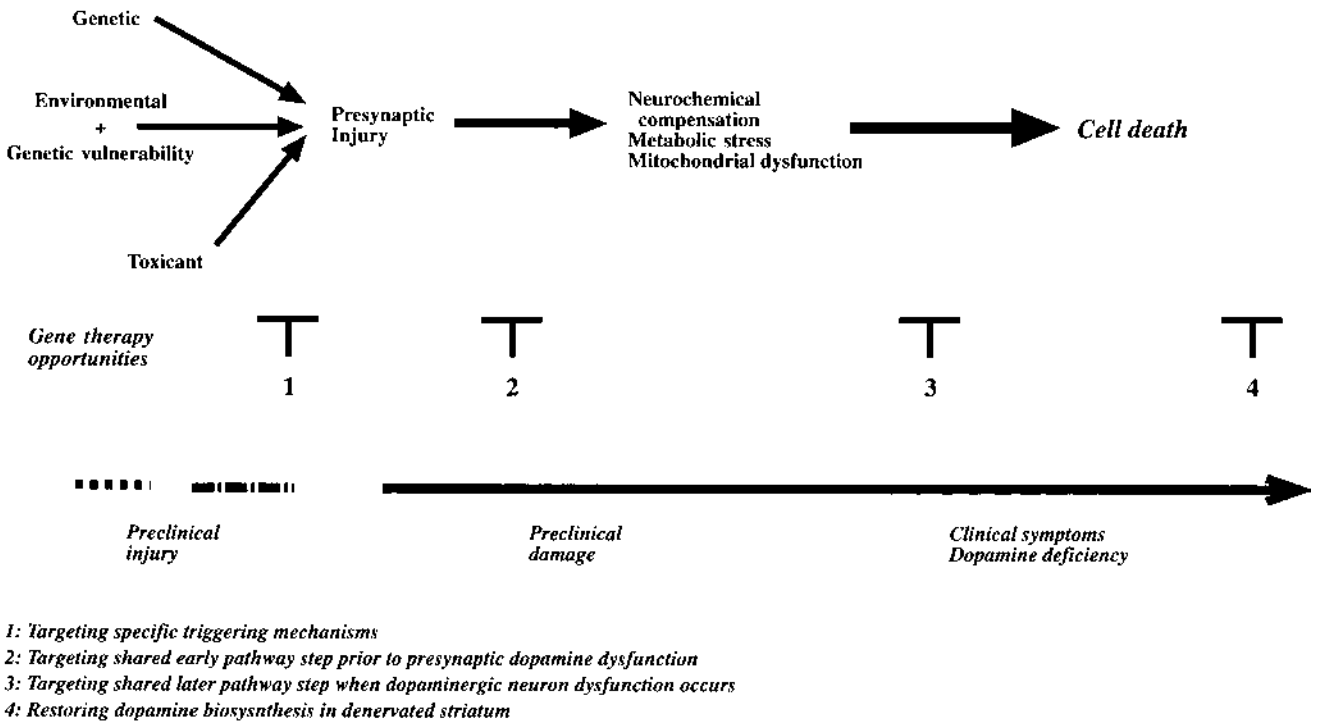

Figure 2 Schematic depicting the common pathway model for PD and points along this pathway where gene-based therapy may be applied. (From Ref. 252, © 2001 Elsevier Science B.V.)

Other groups have implicated a combination of herbicide and fungicide exposure as a potent risk factor for PD (26,28). Mice treated with a combination of paraquat and maneb demonstrated a sustained decrease in motor activity and reduced tyrosine hydroxylase immununoreactivity in the dorsal striatum. These effects were greater in combination than either paraquat or maneb alone, suggesting that multiple compound exposure alone, or in concert with genetic vulnerability, may trigger a cascade of events leading to PD.

3. Genetic Factors

Several genetic factors have recently been identified to play a central role in the etiology of familial PD. In 1996, genetic linkage strategies were applied to a large Italian family with early onset PD (17,30). The susceptibility gene was identified on the long arm of chromosome 4q21–q23 and positional cloning identified a missense mutation in the α-synuclein gene at position 53 (A53T) (31). Another mutation in α-synuclein, A30P, was identified in a German family (32). Although mutant α-synuclein is responsible for a small number of PD cases, it has vaulted to the forefront of PD research since wild-type α-synuclein has been identified as a key component of Lewy bodies. In fact, α-synuclein staining is now widely used as a neuropathological criterion for PD (33–38).

Two other genetic mutations related to proteasome function have been identified in familial PD. One mutation in the *parkin* gene was discovered in an early onset juvenile autosomal recessive form of parkinsonism (AR-JP) that presents with mild symptoms, slow progression, and the absence of Lewy bodies (39–41). Homozygous deletions and point mutations in *parkin* also account for the majority of autosomal recessive inherited PD (42–48). A second mutation in the ubiquitin carboxy-terminal-hydrolase-L1 (UCH-L1) gene has been identified in a German family with PD (49). Both Parkin and UCH-L1 are involved in the regulation of the ubiquitin-proteasome pathway that suggests dysregulation of protein processing in the pathophysiology of PD (21,50,51).

4. Potential Gene-based Therapies for Parkinson's Disease

As outlined in Fig. 1 and discussed above, toxicant and environmental triggers combined with genetic vulnerability are likely to converge on a common pathway toward cell death in PD. These common downstream events that culminate in neuronal cell death are all possible targets for gene therapy in the treatment of PD. Teaming the appropriate gene with the most powerful vector system will make these treatments a clinical reality for PD-afflicted individuals. The following sections summarize the present state of the art for the application of gene transfer technologies to treatment of PD.

a. Ad Vectors and PD. Adenovirus-based therapies for PD have been tested extensively in rodent models. A first-generation Ad vector encoding the TH gene, when introduced into the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats, led to a reduction in amphetamine-induced rotational behavior (52). Vector-directed TH gene expression was observed for 1 to 2 weeks following gene transfer, but was accompanied by a vigorous inflammatory response, gliosis, and local tissue damage. Ad vectors expressing the TGF-β family member, glial cell line-derived neurotrophic factor (GDNF), have also been tested in 6-OHDA-lesioned rats where protection of the dopaminergic phenotype from chemical-induced damage is observed for up to 6 weeks postlesion (53). This protection was equivalent in scope if the vector was delivered to the striatum or the SN prior to the 6-OHDA lesion (54,55). GDNF delivery via an Ad vector was shown to exhibit differential effects in the lesioned aged rat as opposed to younger rats. Connor and colleagues demonstrated that Ad-mediated GDNF delivery was only protective in aged rats when the virus was delivered to the striatum, whereas the dopaminergic system of young 6-OHDA-lesioned rats could be protected by delivery to either the SN or striatum (56). This group hypothesized, based proposals by Zigmond et al., that compensatory changes occur in the CNS of the aged rat that likely increases its sensitivity to Parkinsonian lesioning (57).

b. AAV Vectors and PD. Recombinant AAV vectors exhibit great promise in the arena of PD gene therapy. For example, Kaplitt and others injected AAV vectors expressing either β-galactosidase or human tyrosine hydroxylase (hTH) into the brains of 6-OHDA-lesioned rats and demonstrated long-term transgene expression (3 months) and functional recovery (58). Mandel and colleagues demonstrated longer-term transgene expression (at least 1 year) in the rat striatum (59). In this series of experiments, AAV vectors were constructed to express either hTH or human GTP-cyclohydrolase I (GTPCHI) in the 6-OHDA-lesioned rat striatum. Elevated levels of L-dihydroxyphenlaline (L-DOPA) were observed in animals receiving both vectors, but disappointingly, no reduction in apomorphine-induced rotational behavior was apparent in these animals. CED-mediated delivery of AAV expressing the aromatic amino acid decarboxylase (AADC) gene to the striata of MPTP-lesioned monkeys resulted in L-DOPA-regulated dopamine production and release in these parkinsonian animals (11). An illustration of an experiment from this study is shown in Fig. 3. Another study using AAV vectors to deliver hTH and AADC genes to the brains of MPTP-treated monkeys showed that AAV vectors could direct long-term transgene expression devoid of significant toxicity (60). Recently, Shen and colleagues have used a triple AAV vector administration approach to combat PD (1). These vectors encode for TH, AADC, and GTPCHI, and were shown in combination in rats to enhance levels of tetrahydrobiopterin (BH4) and dopamine production, as well as to improve apomorphine-induced rotational behavior for up to 1 year. Other studies have also demonstrated extended expression in the CNS using AAV vectors (61–63).

A paper by During and colleagues (64) described the use of AAV vectors to express the glutamic acid decarboxylase (GAD) gene GAD65 in the setting of experimental PD to phenoconvert excitatory neurons (glutamate neurotransmitter releasing) to inhibitory neurons (GABA neurotransmitter-releasing). The rationale behind this novel approach relates to the altered neurotransmission that occurs within the basal gan-

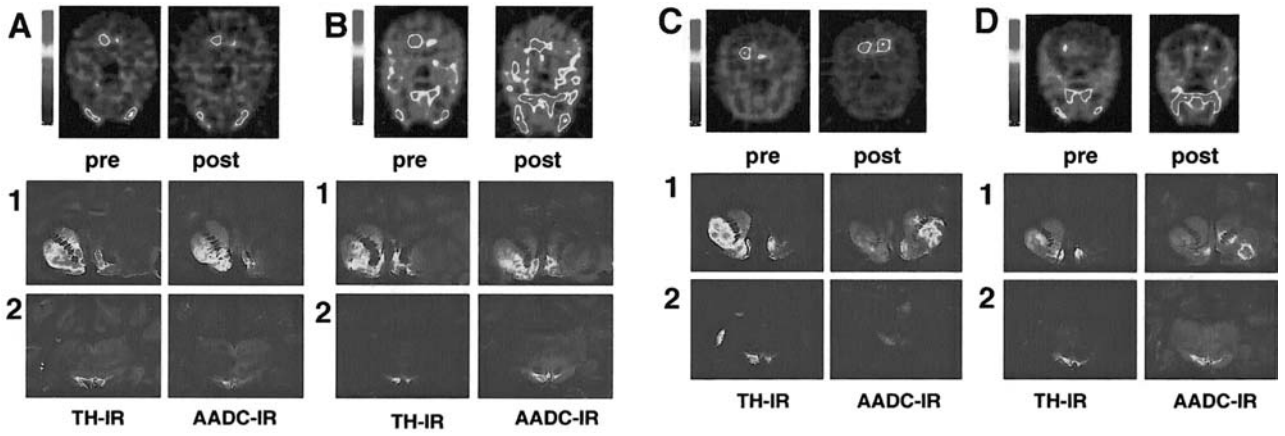

Figure 3 Convection-enhanced delivery of an AAV vector expressing the aromatic L-amino acid decarboxylase (AADC) gene in Parkinsonian nonhuman primates. Rhesus monkeys received the dopaminergic toxin MPTP to establish a stable parkinsonian state. A combined unilateral intracarotid artery and intravenous MPTP injection protocol was performed to produce a nearly complete dopaminergic lesion on the side of the carotid artery infusion (ipsilateral side) and a partial lesion on the other side (contralateral side). Once this was achieved, the animals received ipsilateral infusions of either AAV-AADC or the β-galactosidase control vector AAV-LacZ via convection-enhanced delivery into the striatum. Positron emission tomography studies of uptake of the AADC tracer 6-[18F]fluoro-L-*m*-tyrosine (FMT) in the striatum are shown for AAV-LacZ (upper segments of A and B) and AAV-AADC-injected monkeys (upper segments of C and D) pre- and postviral injection. FMT uptake was negligible in the ipsilateral striatum in all monkeys prior to AAV administration. After virus injection, AAV-LacZ-treated monkeys showed little change in ipsilateral FMT uptake, whereas AAV-AADC-treated animals demonstrated a dramatic increase in ipsilateral FMT uptake. After sacrifice, immunohistochemical analyses for tyrosine hydroxylase (TH-IR) and AADC (AADC-IR) expression were performed on the striatum (row 1 of each panel) and substantia nigra (row 2 of each panel). TH- and AADC-IR were markedly reduced in the ipsilateral striatum of AAV-LacZ-injected monkeys, whereas only AADC-IR was restored on the ipsilateral striatum of AAV-AADC-treated animals. In data not shown, AAV-AADC monkeys exhibited a marked enhancement in dopamine production when the animals were given L-DOPA, indicating that the AADC transgene product was bioactive. (From Ref. 11, © 2000 Elsevier Science B.V.) See the color insert for a color version of this figure.

glia during PD. In this disease, dopaminergic neuron projections from the substantia nigra pars compacta to the striatum degenerate. The observed reduction in dopamine release in the striatum results in reduced GABA neurotransmission, also called ''disinhibition,'' in the subthalamic nucleus (STN). This state, coupled with direct disinhibition within the striatum itself, results in increased glutamate neurotransmission and thus excessive excitation of the substantia nigra pars reticulata (SNr) and the globus pallidum internal segment (GPi). Delivery of AAV expressing GAD65 to the STN led to increased GABA release in the SNr, shifted SNr single-unit recording responses from primarily excitatory to inhibitory, and if delivered prior to 6-OHDA lesioning protected rats from the neurotoxin. These results, although intriguing, require a comprehensive evaluation of long-term effects that neuronal phenoconversion may have on basal ganglia circuitry.

c. Lentivirus Vectors and PD. Lentivirus-based therapies for PD have been tested extensively in rodent and nonhuman primate models. Injection of a self-inactivating (SIN) lentivirus vector expressing GDNF has been shown to be protective in the 6-OHDA rat and MPTP nonhuman primate models of PD and in nonlesioned aged rhesus monkeys (65–67). An example of one of these studies is shown in Fig. 4. Long-term striatal overexpression of this potent factor by a lentiviral vector in nonlesioned rats has recently been shown to markedly down-regulate TH expression (68). The mechanism responsible for GDNF-mediated repression of TH expression is presently not understood, but the observation supplicates caution in implementation of gene transfer approaches clinically that involve long-term, uncontrolled GDNF expression.

Lentiviral vectors have been shown preclinically to be effective with other approaches to treat PD. The dopamine biosynthetic pathway, which is compromised in PD, may require the reintroduction of multiple components to restore physiological levels of dopamine. Due to the moderate size capacity of lentiviral vectors, multicistronic versions of the vector platform have been developed. Azzouz and colleagues recently created a lentiviral vector that coexpresses aromatic L-amino acid decarboxylase, tyrosine hydroxylase, and GTP cyclohydrolase I (69). Delivery of this vector to the striata of 6-OHDA-lesioned rats led to stable dopamine biosynthesis and functional improvement for up to 5 months posttreatment.

d. HSV Vectors and PD. Dependent the transgene size capacity of a given gene transfer vector, the use of cellular promoters to direct transgene expression appears to be another

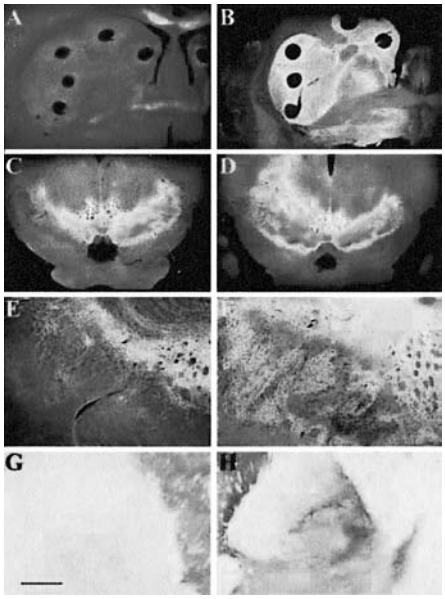

Figure 4 Delivery of a lentiviral vector expressing the glial cell line-derived neurotrophic factor (GDNF) gene in Parkinsonian nonhuman primates. Rhesus monkeys received the dopaminergic toxin MPTP unilaterally (right side) to establish a parkinsonian state. One week later, the animals received ipsilateral infusions of lentiviral vector expressing glial cell line-derived neurotrophic factor (lenti-GDNF) or one expressing the reporter protein β-galactosidase (lenti-βGal). Three months following treatment, the animals were sacrificed and immunohistochemistry was performed. A and B depict low-power, dark-field photomicrographs through the right striatum of TH-immunostained sections of MPTP-treated monkeys treated with lenti-βGal (A) or lenti-GDNF (B). There appeared to be a comprehensive diminution of TH immunoreactivity in the caudate and putamen of lenti-βGal-treated animals, whereas a nearly normal level of TH immunoreactivity was seen in the animals receiving lenti-GDNF. Low-power (C and D) and medium-power (E and F) photomicrographs are shown of a TH-immunostained section through the substantia nigra of animals treated with lenti-βGal (C and E) and lenti-GDNF (D and F). Note the loss of TH-immunoreactive neurons in the lenti-βGal-treated animals on the side of the MPTP infusion. TH-immunoreactive sprouting fibers, as well as an above normal number of TH-positive nigral perikarya, are observed in lenti-GDNF-treated animals on the side of the MPTP injection. G and H depict low-power, bright-field photomicrographs of a TH-immunostained section from a lenti-GDNF-treated monkey. Note the normal TH-immunoreactive fiber density through the globus pallidus on the intact side that was not treated with lenti-GDNF (G). In contrast, an enhanced network of TH-immunoreactive fibers is seen on the side treated with both MPTP and lenti-GDNF. Scale bar in (G) represents the following magnifications: A, B, C, and D at 3500 μm; E, F, G, and H at 1150 μm. (From Ref. 66, © 2000 AAAS.) See the color insert for a color version of this figure.

promising strategy for development of a gene-based therapy for PD because these promoters tend to yield longer-term, cell-specific expression. This has been demonstrated using HSV amplicons equipped with either the preproenkephalin or 9-kb tyrosine hydroxylase (TH) promoter in vivo. Kaplitt and colleagues showed extended expression duration and striatal cell specificity using a version of the preproenkephalin promoter inserted into an HSV amplicon (70). Using 9-kb rat TH promoter to drive expression of the β-galactosidase (*lacZ*) reporter gene in the mouse striatum, Jin et al. observed expression of *lacZ* in TH-positive dopaminergic neurons in the substantia nigra due to retrograde transport of amplicon virions (71). In one of the few published PD-related studies using amplicons as the chosen gene therapy vector, During and colleagues treated 6-OHDA-lesioned rats with an HSV amplicon expressing human TH (72). Behavioral and biochemical recovery was maintained for 1 year following vector introduction.

e. Nonviral Vectors and PD. The use of nonviral means of DNA delivery for treatment of PD is appealing due to the inherent lack of immunogenic and/or cytotoxic viral gene products in the system. Nonviral delivery of genes includes the following means of transfer: ''naked'' DNA, DNA encapsulated within cationic lipids or polycationic polymers, or DNA attached to positively charged metal particles and introduced via particle bombardment (3,73–75). Until recently, successful implementation of this gene transfer modality has largely been impeded by the low transfection efficiencies observed in neurons. In vivo nonviral means of DNA transfer were initially demonstrated to occur in muscle cells with surprising efficiency (76). DNA transfer using polycationic lipid formulations to glia and neurons has been also demonstrated, albeit at low efficiencies (10,73). Martinez-Fong and colleagues recently described their use of a neurotensin-SPDP-poly-L-lysine conjugate that was competent to bind and transfer plasmid DNA to neurotensin receptor-expressing cell lines (N1E-115 and HT-29) (77). Because the high-affinity neurotensin receptor is expressed by a subset of neurons of the nigrostriatal and mesolimbic dopaminergic systems, this nonviral gene delivery modality could prove useful in the treatment of PD. In Addition, the 25-kD cationic polymer polyethylenimine (PEI) was shown by Abdallah et al. to mediate transfer of a luciferase-expressing plasmid to neurons and glia of adult mice (78).

Pardridge and colleagues have developed an interesting nonviral gene delivery modality that has been recently tested in the 6-OHDA rat model of PD (79). A TH-expressing plasmid, encapsulated in PEGylated immunoliposomes (PIL) that were targeted to the brain with a rat transferrin receptor-specific monoclonal antibody, was administered intravenously. The plasmid was shown to effectively cross the blood–brain barrier and lead to transient normalization of TH levels within the striatum of 6-OHDA-lesioned rats. If issues regarding transgene expression silencing can be resolved and strict targeting to the striatum can be achieved, this approach may be one of the more promising applications of nonviral gene transfer-based therapies for PD.

B. Alzheimer's Disease

1. Introduction

As the most common cause of senile dementia, Alzheimer's disease (AD) affects millions of people worldwide. Clinically, patients experience progressive cognitive impairment leading to dementia and ultimately death. The neuropathological hallmarks of AD are senile neuritic plaques (NPs) and neurofibrillary tangles (NFTs). Neuritic plaques are extracellular aggregations of protein, including the fibrillar peptide, β-amyloid. NFTs are neuronal inclusions of filamentous structures containing hyperphosphorylated forms of the microtubule-associated protein tau. Although these tissue and cellular abnormalities are well described, the role of each in producing neuronal demise is still actively debated.

AD is divided into 2 types based on age of symptom onset, early (before 60 years) and late (after 60 years). Early-onset Alzheimer's disease (EOAD) is primarily an inheritable form of the disease. Genetic linkage studies of several families exhibiting EOAD identified a locus on the long arm of chromosome 21 near the amyloid precursor protein (APP) gene (80). Further studies revealed that mutations in APP gene increased the production of β-amyloid (a major constituent of senile plaques) (81). The APP linkage represents $<1\%$ of early onset cases suggesting other genetic loci exist. Two loci, one on chromosome 14q and the other on chromosome 1 containing the presenilin (PS)-1 and- 2 genes, have also been identified in linkage studies (82,83). Many mutations in PS-1 have been identified in EOAD cases, which account for $>50\%$ of all EOAD cases. The function of the PSs is currently emerging and appears to involve signal transduction (84–86).

A genetic locus on chromosome 19q is implicated in late-onset Alzheimer's disease (LOAD) (87,88). The apolipoprotein E gene is located in this region, but no mutations have been found in AD; however, 1 of 3 isoforms of ApoE, E4, is a significant risk factor for LOAD (81). Other potential LOAD-linked genes have been identified (89–95) and further study is needed to substantiate the importance of their linkage. The vast majority of LOAD cases are sporadic with no identified genetic component. Studies using microarray gene expression profile analysis are beginning to identify altered gene expression in LOAD compared with normally aged brains (P. Coleman, personal communication, 2003). Such analyses will lead to further studies characterizing the role of candidate genes in AD, possibly allowing for a genetic screen to identify the best therapeutic strategy.

2. Putative Mechanisms Underlying AD

Based on the neuropathological findings of AD, NPs, and NFTs, extensive research efforts have focused on their role in the pathogenesis of AD. Many hypotheses are under examination: the acetylcholine hypothesis (96,97), the amyloid cascade hypothesis (98–100), the neuroinflammatory hypothesis (101), energy metabolism hypothesis (102), and the oxidative stress hypothesis (103). A comprehensive review of the research behind each hypothesis is beyond the scope of this discussion; instead, each is discussed generally as a potential target area for gene therapy.

3. Potential Gene-based Therapies for AD

One of the affected brain regions is the basal forebrain cholinergic complex as cholinergic deficiency correlates with both the magnitude of pathological severity and degree of dementia (96,104–107). One therapeutic approach is to augment cholinergic function by increasing activity of the biosynthetic enzyme for acetylcholine, choline acetyltransferase (ChAT) to the affected area. Nerve growth factor (NGF) up-regulates expression of ChAT (108), and promotes survival and maintenance of the septohippocampal pathway that is a major pathway for memory and learning (109). Studies reporting that NGF increases β-amyloid production in vitro contrast with in vivo studies, suggesting that NGF delivery does not increase plaque formation in primates (110). Grafting of fibroblasts genetically modified to produce NGF via a Moloney murine leukemia virus vector promotes restoration and survival of the septohippocampal pathway (111–115). Figure 5 depicts an example of the bioactivity of these transduced grafts in vivo. NGF clearly has therapeutic potential but further studies will be needed to evaluate its efficacy in ameliorating symptoms of AD.

Amyloid-containing plaques may be a cause of AD or a byproduct of the disease process. Identified mutations in both the APP and the PS genes correlate with increased production of β-amyloid and potentially plaques. PSs are transmembrane proteins localized predominantly in endosomes and Golgi apparati and are believed to promote the aggregation of β-amyloid by increasing the activity of γ-secretase, the enzyme responsible for liberating the Aβ 1–42 fragment from APP (86,116). Gene products that may modify the activity of β- or γ-secretase or proteins capable of disrupting β-amyloid aggregation may potentially slow progression of the disease.

Other areas of therapeutic interest for AD are inflammation and oxidative stress. Many mediators of inflammation have been detected in the brain of AD brain [reviewed in (101)]. Whether inflammation is activated by the production of plaques and tangles or inflammation initiates production of plaques and tangles, which in turn propagates an inflammatory response is not clear. It is believed that the inflammatory response of glia that leads to neuronal demise (101). Epidemiological studies with antioxidants and nonsteroidal anti-inflammatory drugs suggest that decreasing free radicals and inhibiting inflammatory processes may confer protection against AD and/or slow the rate of cognitive decline seen in AD [discussed in (103)]. The delivery of gene products capable of scavenging free radicals and blocking inflammatory processes may also prove an effective therapeutic approach.

Recent publications have highlighted the potential of active and passive Aβ-based immunotherapies in the treatment of AD (117–119). Numerous investigative teams have reported diminution in AD-like pathology (i.e., Aβ deposition) and behavioral improvements in different animal models of the disease as a result of Aβ peptide immunization or via the administration of Aβ-specific antibodies. The biological

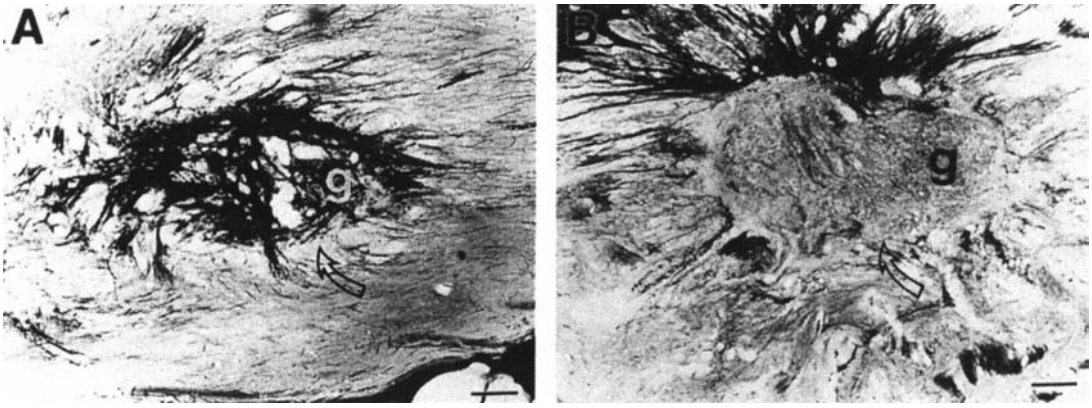

Figure 5 Neurite penetration into implanted primary fibroblast cells that have been transduced with a murine retrovirus vector expressing nerve growth factor. (A) depicts an NGF-secreting autologous fibroblast graft located in a region of intact Nucleus Basalis of Meynart (NBM) showing penetration by acetylcholinesterase (AChE)-positive neurites 1 month after grafting in cynomolgous monkey. Arrow and ''g'' indicate graft. In contrast, an implanted NBM graft initially transduced with a control β-galactosidase-expressing retrovirus vector (B) exhibits no penetration of AChE-positive neurites, although sprouting at the edge of the graft potentially due to astrocytic NGF secretion is apparent. These studies and many others suggested that NGF-expressing autologous fibroblast grafts might represent a viable approach to promote regeneration of cholinergic neuronal pathways targeted by Alzheimer's disease. Early-phase human clinical trials are in progress to determine the safety of this therapeutic methodology. (From Ref. 114, © 1994 Elsevier Science B.V.)

mechanism(s) by which these therapies act are still subject to interpretation. However, 2 nonmutually exclusive hypotheses have emerged: (1) Aβ-specific antibodies whether raised intrinsically or passively administered act at the blood–brain barrier (BBB) to alter the equilibrium of soluble $A\beta_{1-42}$ concentrations to favor its clearance from the brain. This is posited to prevent additional Aβ peptide deposition and perhaps promote aggregate dissolution. (2) Aβ-specific antibodies cross a compromised BBB and bind to Aβ within the brain parenchyma where antibody-Aβ complexes are postulated to be bound by complement, and recognized by microglia, which dissolve existing amyloid deposits. Both mechanisms of vaccine action can lead to possibly harmful side effects, with the most feared being systemic autoimmune disease and CNS inflammation as illuminated by recent findings from a Elan Pharmaceuticals peptide vaccine phase II clinical trial conducted in Europe [commentary in (120,121)]. Although presently untested, gene transfer technology due to its inherent versatility may allow for regulated antigen presentation and even codelivery of immunomodulatory gene products that could lead to safer and more efficacious vaccines for AD.

C. Lysosomal Storage Diseases

1. Introduction

Lysosomal storage diseases (LSDs) are a diverse group of greater than 40 disorders that originate from a deficiency of a lysosomal enzyme, resulting in accumulation of lysosomal proteins and cellular dysfunction. Many of the LSDs exhibit moderate to severe deleterious effects on somatic tissues as well as the CNS. These degenerative disorders differ from many of the neurodegenerative diseases described in that the pathogenic mechanism responsible for most LSDs is already known (i.e., lysosomal enzyme deficiency, defects in cofactors or transport proteins). There are examples of LSDs, however, for which the missing lysosomal function/protein activity has not been identified [e.g., a subset of the ceroid lipofuscinosis diseases; reviewed by (122)]. Depending the type of LSD, enzyme replacement by direct infusion of the missing enzyme into peripheral tissues has been an extremely successful strategy (123). This is no more evident than in the case of Gaucher disease, which results from a deficiency of glucocerebrosidase. Loss of glucocerebrosidase leads to accumulation of glucocerebroside, a byproduct of sphingolipid degradation, in macrophages resulting in spleen and liver enlargement, bone malformation, and pulmonary dysfunction (123). Infusion of recombinant glucocerebrosidase leads to significant correction of peripheral tissue disease (124). A similar approach has proven successful in the treatment of Fabry disease, another sphingolipid disorder that primarily affects kidney, heart, and skin [reviewed by (125)]. In clinical trials, intravenous delivery of α-galactosidase A was found to be safe and led to marked reductions in plasma glycosphingolipid levels and microvascular endothelial deposits in major organs (126,127).

Other LSDs exhibit both peripheral and CNS involvement. The CNS component of the mucopolysaccharidosis (MPS) storage disorders and other LSDs has proven a more difficult task to correct using standard enzyme replacement therapy. This difficulty lies in the inability of peripherally infused lysosomal enzymes to traverse the blood–brain barrier. Development of methodologies to effectively deliver and distribute

the deficient enzyme throughout the brain would represent a major advance in therapies for LSDs. To that end, the implementation of gene-based technology in this endeavor has been enthusiastically pursued and has demonstrated initial promise.

2. Potential Gene-based Therapies for Correcting CNS Dysfunction Caused by LSDs

Numerous mouse models for the various LSDs have been developed and have proven extremely useful in assessing novel therapies. Gus^{mps} mice exhibiting the β-glucuronidase enzyme deficiency associated with Sly disease undergo progressive lysosomal accumulation of non-egraded glycosaminoglycans in multiple organs including the brain. The Sands laboratory demonstrated that AAV vector-mediated delivery of β-glucuronidase into the anterior cortex and hippocampus of newborn MPS VII mice led to a reduction in glycosaminoglycan deposition and concomitant improvement in cognitive function as measured in the Morris Water Maze paradigm (128). Although transgene expression levels were generally greater than normal β-glucuronidase levels, particularly at the injection site, no overt toxicity was observed. In addition, these results suggested that a single point source of continuous enzyme expression produced lysosomal storage correction at distal sites. Several other groups have also demonstrated the utility of AAV vector-mediated β-glucuronidase delivery in gus^{mps} mice (129–131).

Davidson and colleagues have used recombinant viral vectors based on feline immunodeficiency virus (FIV) to deliver the β-glucuronidase gene to the brains of MPS VII mice to determine if restoration of this enzyme diminished preestablished lysosomal accumulations and corrected associated CNS deficits (132). FIV vector-mediated bilateral delivery of β-glucuronidase via the striatum led to bilateral correction of protein deposits and a reversal of spatial learning and memory impairments. The effect FIV vector-mediated expression of β-glucuronidase on lysosomal storage is illustrated in Fig. 6. Perhaps shedding light on potential mechanisms of action, gene expression profiling indicated significant increases in genes associated with neuronal plasticity mediation. An interesting extension of these studies was performed recently by Elliger and colleagues (133). The coding sequences for the Igκ secretion and HIV-1 TAT uptake signals were engineered into the β-glucuronidase open reading frame in order to effect therapeutic benefit in more distal organs. This modified transgene was delivered intrathecally via an AAV recombinant vector to newborn gus^{mps} mice. Treated mice were found to be more active, exhibited less stunted growth, and did not show evidence for abnormal storage deposits in the brain or liver, or in tissues not harboring AAV vector genomes.

The Twitcher mouse, which harbors a genetic disruption in the galactocerebrosidase locus, serves as an informative murine model for human globoid cell leukodystrophy (Krabbe disease). Intraventricular infusion of a recombinant adenovirus vector expressing galactocerebrosidase to newborn Twitcher mice led to marked reduction in lysosomal storage pathology (134). However, unlike in the case of gene delivery to MPS VII mice, treatment of Twitcher mice with preestablished disease had no significant effect on disease pathology. These results indicate that timing of interventions for Krabbe disease is crucial, and/or adenovirus vectors, due to their inherent immunogenicity and reduced duration of expression, are not as useful for treating this LSD. It is interesting to note that there exist autosomal recessive forms of Krabbe disease that have been identified in dogs and rhesus monkeys (135). As other gene transfer vector approaches are applied to this disease, these higher order mammalian models will become invaluable in the stepwise progression toward clinical application to the human form of Krabbe disease.

D. Huntington's Disease

1. Introduction

Huntington's disease (HD) is a fully penetrant genetic neurodegenerative disorder that is inherited as an autosomal dominant mutation of the *HD* gene (136). Affected individuals begin to exhibit symptoms in the third to fifth decade of life with some cases of juvenile (under 20 years old) and late onset (over 65 years old) (137,138). Clinical manifestations of the disease include progressive chorea, emotional disturbances, and dementia. Neuropathological features of HD include an extensive loss of neurons and astrogliosis in the striatum, primarily the caudate nucleus. Histologically, intracellular inclusions consisting of ubiquitinated polyglutamine aggregates have been found in neurons of the striatum and other less affected areas such as neocortex.

The *HD* gene encoding the huntingtin protein was first localized to chromosome 4p16.3 in 1983 (139). The normal function of huntingtin remains an enigma; however, cloning of the normal and diseased gene revealed the mutant form of the gene contains an increased number of glutamine encoding CAG repeats in the amino terminus (139). Normal individuals contain 11 to 34 repeats, whereas affected individuals contain 40 or greater repeats (140,141). A study by Ashizawa et al. (141) provided evidence that repeat numbers correlated with age of onset and clinical symptom severity; however, the mechanism by which the expression of this mutant protein leads to cell death remains elusive.

2. Possible Mechanisms of Disease

Understanding the normal function of huntingtin may prove pivotal in understanding how the mutant form leads to the selective loss of medium spiny neurons in the striatum. In the absence of this knowledge, studies have suggested possible mechanisms by which the mutant HD gene product causes cell death and provided a basis for developing therapeutic strategies.

One proposed mechanism involves a pathway of cellular protein degradation. The ubiquitin/proteasome pathway is the major protein degradation pathway of the cell. The proteasome, a cylindrical peptidase-containing complex that cleaves ubiquitinated proteins into their amino acid constituents, is believed to be involved in the degradation of huntingtin (142,143). In Huntington's disease, ubiquitinated polyglutamine aggregates have been identified, suggesting the digested

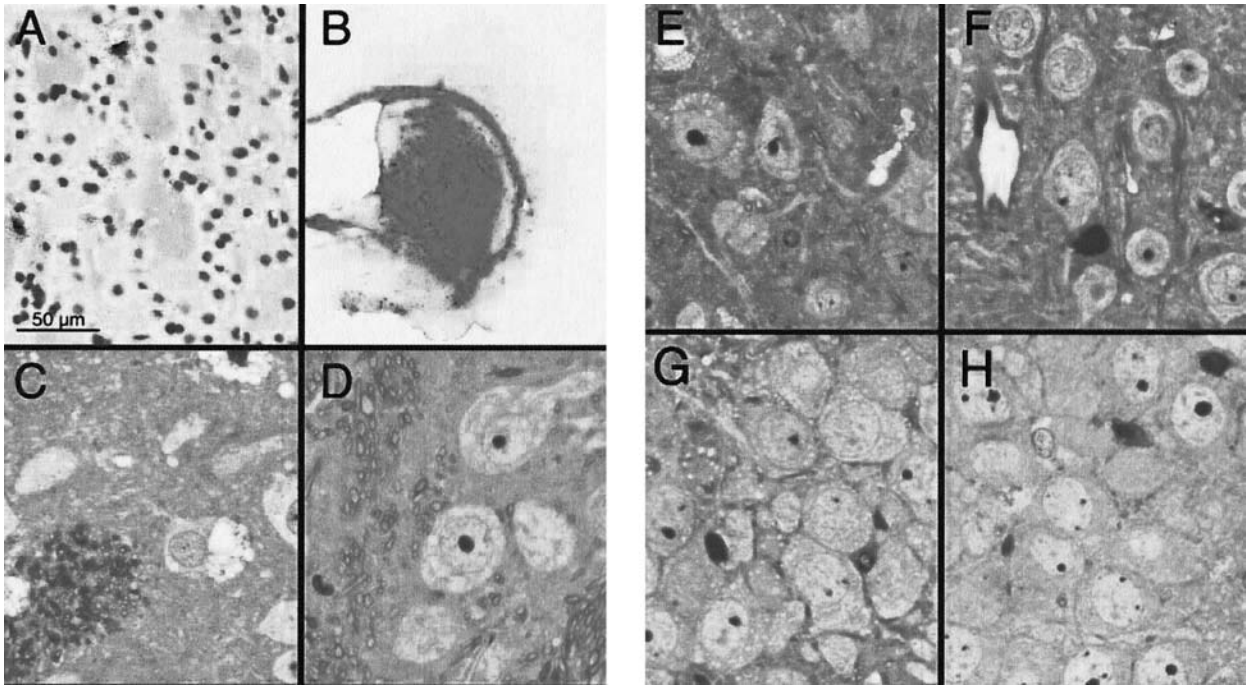

Figure 6 β-Glucuronidase expression following FIV-mediated gene transfer into the striata of MPS VIII mice and its effect on lysosomal storage. Eight-week-old MPS VII (gus^{mps}) mice were injected unilaterally into the striatum with 1×10^6 transduction units of a feline immunodeficiency virus (FIV) vector expressing β-glucuronidase (FIVβgluc). Six weeks later the animals were sacrificed and analyzed for transgene expression, β-glucuronidase enzyme activity, and lysosomal storage profiles. Transgene-positive cells were detected near the injection site as revealed by in situ RNA analyses (A). β-Glucuronidase activity in the brain of a MPS VII mouse injected with FIVβgluc was found to encompass a wide volume of the brain as determined by histological staining (red staining; B). Representative examples of lysosomal storage in the striatum (C), cortex (E), and hippocampus (G) in nontreated 8 to 12-week-old MPS VII mice are shown. Noticeable lysosomal storage is evident at this age. Analysis of age-matched, FIVβglu-treated MPS VII mice shows significant correction of the storage deficit in the contralateral striatum (D), cortex (F), and hippocampus (H). In data not shown, treated animals exhibited improved learning and memory behavior, indicating that lentivirus-based delivery of β-glucuronidase to the CNS of an animal with preestablished lysosomal storage disease can reverse the neurological deficits caused by the disease. (From Ref. 132, © 2002 National Academy of Sciences.) See the color insert for a color version of this figure.

mutant form of huntingtin creates aggregates leading to an apparent apoptotic cell death (144,145).

Huntingtin has been shown to interact with huntingtin-associated protein 1 (HAP-1) (146), huntingtin-interacting protein 1 and 2 (HIP-1, -2) (147,148) glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (149), and calmodulin (150). HAP-1 binding to huntingtin is enhanced by increased glutamine repeat length (146). It is believed to be involved in vesicular membrane trafficking, but this has not been proven (151). Unlike HAP-1, HIP-1 binding to huntingtin is inversely related to the polyglutamine residue number. The loss of this interaction in a HD neuron may affect the cytoskeletal architecture given HIP-1's similarity to cytoskeletal proteins (148). HIP-2 is an ubiquitin-conjugating enzyme that may be involved in the proteasomal degradation or aggregation of mutant huntingtin (152). The glycolytic enzyme, GAPDH, binds to cleaved huntingtin fragments in vitro (149). The increased binding of GAPDH to cleaved huntingtin fragments may alter cellular energy production, leading to membrane depolarization, increased intracellular calcium, and cell death. Huntingtin can from a complex with calmodulin in a Ca^{2+}-dependent manner, but the mutant form binds independent of calcium (150). Subsequent activation of downstream targets of calmodulin, such as those activated in excitotoxic cell death, may lead to cell death. Further understanding of the interactions of huntingtin with the aforementioned proteins or other proteins will allow for targeted gene therapy. Based on our current understanding, therapies designed to block apoptosis, relieve oxidative stress and prevent the expression and accumulation of degraded mutant huntingtin may help to relieve or prevent symptoms in affected patients.

3. Potential Gene-based Therapies for HD

Yamamoto et al. (153) demonstrated that continuous expression of mutant protein is necessary to maintain intracellular

inclusions and symptoms. A therapy designed to decrease mutant protein levels by antisense therapy may prove useful. In this strategy, a viral vector expressing an antisense RNA or ribozyme (154) to huntingtin could be delivered to the striatum. Alternatively, if a technology were available to reduce the glutamine repeats and/or replace the mutant allele with a normal allele, a permanent reversal of the disease might be accomplished. The use of chimeroplasts, target-specific RNA/DNA oligonucleotides, could possibly allow correction of the genetic deficit, but this may not be technically feasible for such a large mutation (155–157).

A second approach takes advantage of the well-characterized neurotrophic factors, endogenous soluble proteins that regulate survival, growth, morphological plasticity, or synthesis of proteins for differentiated functions of neurons (157). The delivery of the neurotrophic factors GDNF, BDNF, or CNTF have shown neuroprotective effects when given prior to a quinolinic acid challenge in rodent and primate models of HD (158–160). The neuroprotective effect of lentiviral vector-mediated delivery of CNTF in the quinolinic acid model for HD is illustrated in Fig. 7. Further analysis is needed to evaluate the full potential of neurotrophic factor treatment for HD.

A third approach would be to target mechanisms of apoptotic cell death and oxidative stress. Similar to neurotrophic factors, this approach may delay the progression of the disease, but does permanently correct the genetic deficit. The antiapoptotic gene, *bcl-2*, has been shown to inhibit neuronal apoptosis both in vitro and in vivo (161–171). Delivery of the *bcl-2* gene to affected neurons may prevent or delay cell death that occurs in HD. If oxidative free radicals contribute to cell death, the expression of gene products capable of directly or indirectly lowering or removing free radicals [e.g., superoxide dismutase (SOD), catalase, glutathione reductase (GR), glutathione peroxidase (GPO), and glutathione (GSH)] might prove useful in combating neuronal loss.

HD is one of a family of trinucleotide repeat disorders, which includes spinal and bulbar muscular atrophy, spinocerebellar ataxia (types 1, 2, 3, 5, and 7), and dentatorubropallidolusian atrophy. Elucidating the mechanisms by which the mutant form of huntingtin leads to disease may aid in the development of successful gene therapies for HD and possibly other related trinucleotide repeats disorders.

E. Stroke

1. Introduction

Hypoxic injuries, such as stroke, are the genesis of substantial morbidity and mortality in neonates and older individuals. Oxygen deprivation can lead to profound effects on motor function and cognition (172), leading to severe disability and diminished quality of life. Stroke is most commonly the result of embolic obstruction that results in either reduced or complete loss of blood flow to downstream fields. Two types of cell death appear to occur following a stroke. At the ischemic core, necrosis is readily apparent and has been linked to elevated extracellular glutamate and intracellular calcium levels. Necrotic cell death involves nonspecific DNA degradation, nuclear pyknosis, diminished membrane integrity, and mitochondrial swelling. Neuronal degeneration within this area following blood vessel obstruction occurs very rapidly (minutes) following the ischemic insult, and due to this time limitation, does not represent a viable target for gene-based therapeutics. In brain areas more distal to the ischemic core, termed the prenumbra, neurons undergo a more delayed form of cell death (hours to days) that exhibits dependence6 *de novo* gene expression (173–175). This delayed neuronal death, or

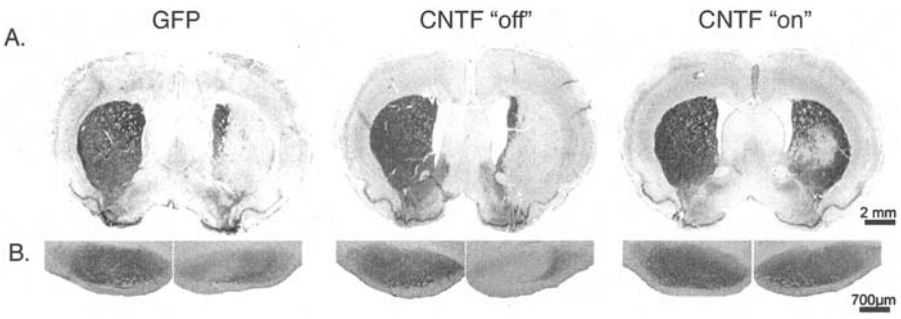

Figure 7 Use of a tetracycline-regulated lentiviral vector expressing ciliary neurotrophic factor (CNTF) in the quinolinic acid rat model of Huntington's disease has a dose-dependent neuroprotective effect. Rats received intrastriatal injections of a tetracycline-regulated lentiviral vector expressing either CNTF (TRE-CNTF) or the reporter protein GFP (TRE-GFP). The vector-injected rats were treated with saline (''off'') or doxycycline (''on'') to regulate transgene expression. Quinolinic acid was infused into the striatum of these mice and they were subsequently sacrificed and brains analyzed by immunocytochemistry for the striatal marker DARPP-32. Representative photomicrographs showing DARPP-32-immunostained striatal (A) and nigral sections (B). The quinolinic acid-induced lesion is clearly indentified by the loss of DARPP-32 staining in the GFP- and CNTF-off groups, whereas a significant protective effect is observed when CNTF expression is switched on. (From Ref. 253, © 2002 Mary Ann Liebert, Inc.)

apoptosis, is capable of producing damage equivalent to the acute necrotic lesion observed soon after severe ischemia. Cells undergoing apoptosis exhibit characteristic fingerprints, including chromosome condensation, internucleosomal DNA fragmentation, and membrane blebbing. A thorough understanding of the gene expression profiles and signaling events that occur in the prenumbra immediately following an ischemic event is likely to yield several molecular/cellular targets that are amenable to pharmacological and gene-based therapeutics.

2. Possible Mechanisms of Disease

Ischemia promotes adaptive and pathologic responses in the neuronal compartment. As illustrated in Fig. 8, extrinsic and intrinsic perturbations in the ischemic brain appear to activate a neuronal ischemic sensor, which in turn promotes adaptive and pathologic gene expression. It is the complex interplay among the numerous molecular signaling cascades and time sensitivity of the disorder that makes development of gene-based approaches for stroke both an exciting and daunting task. The ischemic sensor activates a series of transcription factors that, depending their respective stoichiometry and subcellular localization, act to initiate either an adaptive or pathologic signaling cascade. Insights into candidate gene products that confer protection against ischemic exposure can be gleaned from prior preconditioning studies (176). The gene products involved in adaptive processes include those that inactivate reactive oxygen species (ROS), and those that participate in DNA repair and cytoplasmic calcium regulation (177,178). Hypoxic insult also stimulates the secretion of factors like erythropoietin (EPO) and vascular endothelial growth factor (VEGF), which can act via a paracrine manner to effect neuroprotection (179,180). Other gene products, including heat shock protein 72 (HSP 72) and glucose transporter Glut-1, have been identified that block the initiation of pathologic gene expression (181–184). Such factors may provide sufficient protection during the early stages of cellular responses to ischemia in order to subvert potential downstream apoptotic signaling.

3. Potential Gene-based Therapies for Stroke

Many early experimental gene therapy approaches targeted either intermediate or the late stages of the apoptotic process. The commitment to apoptosis, which is believed to involve mitochondrial permeability transition, can be averted by overexpression of a subset of Bcl-2 protein family members and a neuronal apoptosis inhibitory protein (NAIP). HSV-based vectors overexpressing Bcl-2 reduces the incidence of apoptosis and improves neuronal survival both in vitro and in vivo (167,185). Adenoviral vector-mediated expression of NAIP resulted in reduced levels of activated caspase-3 and diminished neuronal degeneration following transient forebrain ischemia (186,187). Other strategies involve inhibition of cellular caspases that act during later stages of apoptosis through the use of cell-permeable peptides to block the morphologic features of apoptosis (173). In addition, vector-mediated expression of anti-inflammatory molecules (i.e., interleukin-1 receptor antagonist) has been employed to minimize poststroke inflammatory responses within the brain (188–190). However, because ischemia induces global disruptions of cellular processes upstream of mitochondrial commitment, caspase activation, and resultant inflammation, it is unclear whether these strategies can effectively interrupt the initiation of apoptosis or restore function to neurons endangered by this cellular process (191,192). It is also important to note that many of the stroke-related gene transfer approaches performed to date have been employed prior to the onset of experimental ischemia. For gene transfer to be clinically applicable for ischemic disorders, treatments must be assessed following the ischemic event (realistically, at times 1–2 postinjury) (193).

Gene-based neuroprotective approaches that employ neurotrophic and angiogenic factors have also been extensively examined. Tsai and colleagues have used AAV vectors to express GDNF in the setting of experimental stroke as an approach to minimize neuronal damage caused by transient ischemia (194). Rats receiving AAV-GDNF immediately following bilateral common carotid artery ligation and middle cerebral artery occlusion exhibited significantly reduced infarct volumes as compared with animals receiving a β-galactosidase-expressing AAV control vector (Fig. 9). Zhang et al. performed studies using adenovirus-expressed GDNF to examine the therapeutic window following transient middle cerebral artery occlusion (MCAO) in rats (193). The protective effect afforded by GDNF overexpression (i.e., reduced infarct size and inhibition of caspase-3 expression) was evident only if Ad-GDNF was administered at the time of reperfusion, but the effects of the gene transfer were minimal at 1 postreperfusion. These results underscore the importance of early postischemia interventions in minimizing neurodegeneration.

Restoration of energy stores in hypoxic neurons via overexpression of the rat glucose transporter, GLUT-1, has been an approach that has shown promise in experimental models of ischemia and brain injury. HSV vector-mediated delivery of GLUT-1 in 3 models of injury (transient focal cerebral ischemia, kainic acid, and 3-acetylpyridine) led to localized increased uptake of glucose, reduced neuron loss, blunted decline in ATP concentrations and metabolism, and decreased glutamate release and cytosolic calcium levels (183,184,195). Approaches that more directly address the detrimental cytosolic calcium excess observed following ischemia include overexpression of calcium-binding proteins. Viral vector-mediated delivery of the calcium-binding protein calbindin D28K exhibits calcium-buffering activity and is protective in conditions of hypoglycemia and experimental stroke (196–198).

During times of cellular stress, resident proteins can become misfolded, a process that can result in diminution of protein function/activity and potentially lead to intracellular aggregation [reviewed in (199)]. Stress response factors, such as heat shock proteins, have been shown to act as molecular chaperones to assist in protein folding and may represent another approach to support a cell under ischemic attack. Several reports of vector-mediated overexpression of heat shock pro-

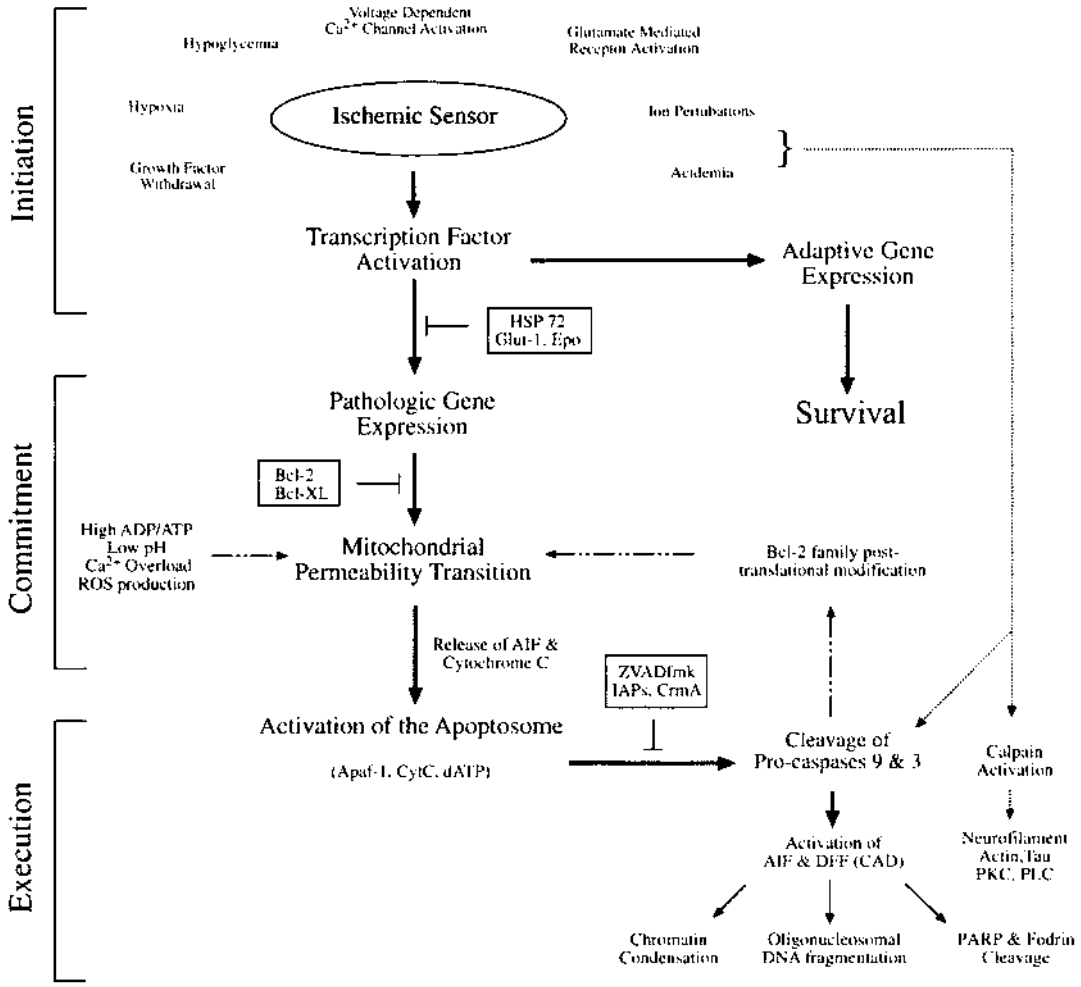

Figure 8 Ischemia promotes adaptive and pathologic responses in the neuronal compartment. Extrinsic and intrinsic perturbations in the ischemic brain activate a neuronal ischemic sensor, which in turn promotes adaptive and pathologic gene expression (solid lines). In addition, select stimuli can activate neuronal death independent of de novo gene expression (dashed lines). Specific gene products, which have demonstrated the ability to block the initiation, commitment, or execution phases of the programmed death pathway, are also included (boxed items). (From Ref. 254, © 1999 Elsevier Science B.V.)

teins (e.g., HSP72) in ischemic animal models demonstrate a neuroprotective role for these factors (200,201). HSV amplicon-mediated delivery of HSP72 to rats 30 min after MCAO resulted in higher numbers of surviving neuron numbers as compared with animals receiving a β-galactosidase-expressing amplicon (200).

In aggregate, gene transfer applications for stroke have shown preclinical promise. For such approaches to gain merit as viable treatment options in humans, major issues need to be addressed. One consideration relates to means of vector delivery. Focal administration of viral vectors following an ischemic event will likely have minimal benefit due to the large areas of the brain that are typically affected. Convection-enhanced delivery may provide the means in which to widely distribute a given therapeutic vector, but remains largely untested in ischemic paradigms (11). A second issue that is not entirely unrelated to the first concerns the window of therapeutic opportunity. As described above, a multitude of signaling events occurs immediately after ischemia that determine whether the compromised neuron follows an adaptive or a pathologic set of molecular instructions. This time window is extremely limited (1–2 poststroke), which makes the implementation of stereotactic means of gene therapy vector delivery nearly improbable. A comprehensive understanding of the molecular signals and their temporal expression profiles will likely identify targets at the earliest of times within this restricted therapeutic window. Until these gaps in disease process knowledge are filled and technical hurdles overcome,

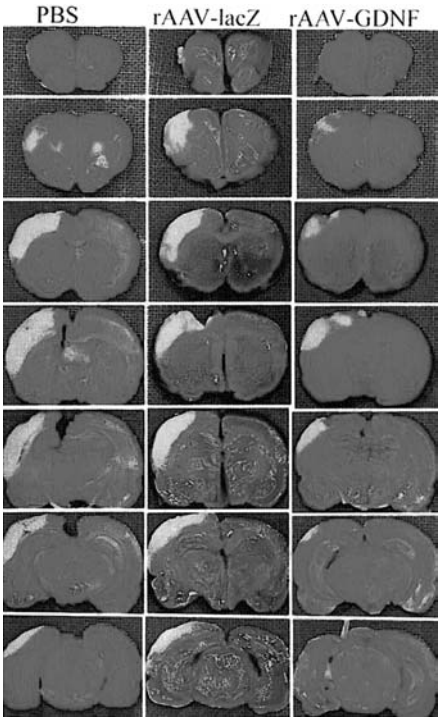

Figure 9 Injection of rAAV-GDNF markedly reduces cortical infarction induced by middle cerebral arterial ligation in rats. The right middle cerbral artery and bilateral common carotid arterial were occluded for 90 minutes. Animals received a PBS, rAAV-lacZ (10^{10} viral particles), or rAAV-GDNF (10^{10} viral particles) unilateral infusion during arterial occlusion, were sacrificed 72 hours later, and their brains were coronally sectioned (2-mm thickness) for TTC staining. White areas represent infarcted zones in the cerebral cortex. Rats receiving rAAV-GDNF exhibited a marked reduction in infarct size as compared to animals receiving the rAAV-lacZ or PBS control. (From Ref. 255, © 2000 Elsevier Science B.V.) See the color insert for a color version of this figure.

clinical gene therapy interventions for stroke will remain an impractical potential alternative to pharmacologic compound-based therapeutics.

F. Epilepsy

1. Introduction

The epilepsies constitute a group of neurological disorders characterized clinically by various seizure syndromes and at the cellular level by hyperexcitability. The inherent cell biological problem is that of pathologic shifting of the normally well-regulated balance between inhibition and excitation toward the latter. The etiology of acquired epilepsy is unknown, although an emerging viewpoint is that early lifetime events, perhaps subclinical in nature, contribute to or trigger the development of the neural substrate underlying the hyperexcitable state [reviewed in (202)]. Because the processes responsible for inducing epileptogenesis are not fully elucidated, present treatment strategies are therefore directed at controlling symptoms (seizures). The seizures are the clinical manifestations of aberrant hypersynchronization of neural networks that can remain local, spread to other locations, and/or involve all cortical regions at once. Neocortical or hippocampal circuits are uniformly activated during an epileptic seizure. Synaptically connected regions may be recruited in a manner to augment or attenuate the discharge. When epileptic discharges travel unimpeded through neural networks, the network functions are subverted, temporarily rendering them incapable of executing normal tasks. With increasing duration of disease, particularly in epilepsies involving the temporal lobe, the neural substrate scars, presumably from chronic exposure to excitatory neurotransmitters (e.g., mesial temporal sclerosis).

For those epilepsies that are unresponsive to pharmacologic therapies, the only present means of relief for some individuals is neurosurgical resection. Due to the highly complex nature of the mammalian CNS and the unknown etiology of epilepsy, the execution of complicated neurosurgery to treat this debilitating disorder is approached with extreme caution. Such medically refractory and surgically difficult cases may prove to be suitable targets for gene therapy. Gene transfer would provide a means to locally alter synaptic transmission in a way as to synchronize excitatory and inhibitory signals, and in doing so, disrupt the hyperexcitable state. Of course, a number of considerations must be addressed, including the cell types to be targeted, localization of the transgene product (e.g., cell intrinsic vs. secreted), transgene expression control (e.g., constitutive vs. regulated), and effects on neighboring normal neuronal circuitry. However, in recent years, novel approaches for treating epilepsy using gene-based means have begun to exhibit promise. Two such preclinical studies are summarized below.

2. Potential Gene-based Therapies for Epilepsy

The development of animal models for epilepsy has greatly enhanced the means in which to assess the possible etiology, and in turn, new therapies for this disorder. Extant in the literature is the observation that accumulation of N-acetyl-L-aspartate (NAA) in the brain leads to induction of seizurelike syndromes. NAA is found predominantly in neurons of the CNS and has served as a useful marker of neuronal injury and/or death in several neurodegenerative diseases. Intraventricular infusions of NAA produce absencelike seizures with signature hippocampal electroencephalograms (EEGs) (spike wave like complexes) in rats (203). A deletion mutation in the aspartocyclase (ASPA) gene, which encodes for the enzyme responsible for metabolizing NAA into aspartate and acetate in glial cells, has been discovered in tremor rats, a model of human petit mal epilepsy (204). The tremor rat model represents a useful in vivo system in which to test novel antiepilepsy gene transfer approaches (205). Seki and colleagues used a

replication-defective recombinant adenoviral vector to deliver the rat ASPA gene to the tremor rat (206). Injection of the ASPA-expressing vector into the brains of 7-week-old tremor rats significantly reduced the appearance of absencelike seizures, as compared with rats receiving a β-galactosidase expressing control adenovirus vector (Fig. 10).

Another animal model that can be used to assess novel gene-based therapies for epilepsy is the protein-L-isoaspartyl methyltransferase (PIMT)-deficient mouse (207,208). PIMT is a ubiquitously expressed enzyme that repairs proteins that have undergone isomerization of aspartate residues or deamidation of asparagine residues. Failure to repair these spontaneously occurring protein alterations leads to mislocalization and diminished protein function (209). In fact, PIMT-deficient mice, which are devoid of both splice variant forms of PIMT, accumulate isoaspartate (IsoAsp) within their brains. One obvious phenotype of this deficiency is the progressive development of fatal epileptic seizures. Intraventricular delivery of both splice variants (PIMT-I and -II) via *exo-utero* means to E14.5 PIMT-deficient mice led to improved growth, diminished IsoAsp accumulation, enhanced survival times, and marked reduction in occurrence of epileptic seizures (210). Sustained therapeutic gene expression and concomitant clinical benefit was evident at times up to 7 weeks postinjection. In aggregate, the promising results garnered from these 2 epilepsy gene transfer studies evoke enthusiasm for further development of gene-based therapies for those epileptic syndromes refractory to standard symptomatic treatments.

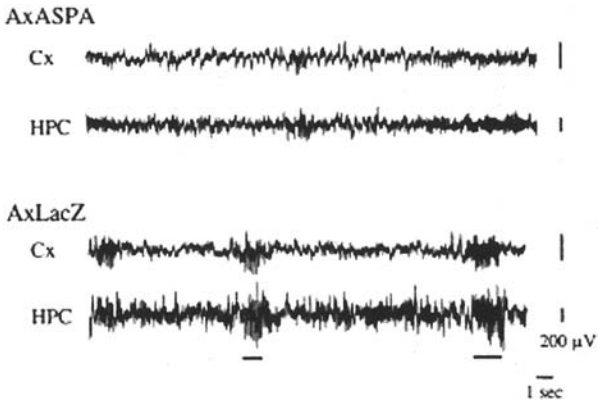

Figure 10 Electroencephalograms (EEGs) recorded from the left frontal cortex (Cx) and left hippocampus (HPC) of tremor rat 1 week after intracerebroventricular administration of an adenoviral vector expressing aspartocyclase (AxASPA) or β-galactosidase (AxLacZ). Lines under EEG represent 5–7 Hz spike-wave-like complexes that lasted for more than 1 second, considered to absence-like seizures. Delivery of AxASPA markedly diminished the occurrence of absence-like seizures in tremor rats, in constrast to animals receiving the control vector AxLacZ. (From Ref. 206, © 2002 Elsevier Science B.V.)

G. Motor Neuron Disease

1. Introduction

The motor neuron diseases are a group of disorders characterized by predominant degeneration of the motor neurons. The most commonly recognized are familial spastic paraplegia (FSP) (upper motor neuron disease); spinal muscular atrophy (SMA) and spinobulbar muscular atrophy (SBMA, or Kennedy's disease) (lower motor neuron diseases); and amyotrophic lateral sclerosis (ALS) (combination upper and lower motor neuron disease). These disease syndromes can range in severity from debilitating (spastic paraplegia; Kennedy's disease) to lethal (some spinal muscular atrophies; amyotrophic lateral sclerosis).

The clinical features of these conditions are well known to neurologists, but the recognition of the molecular basis, and the possibility of molecular confirmation of diagnosis, has clarified the extent and relationship of some of these conditions. A genetic basis has now been identified for all these diseases. Different genetic mechanisms, including missense mutations (ALS), gene deletions or aberrant gene splicing (SMA), and trinucleotide repeat expansions (spinobulbar muscular atrophy), are responsible for the pathologic findings and clinical presentations of these motor neuron disorders. The molecular findings are of variable value as diagnostic tests. More important, it is hoped that understanding the underlying molecular basis of these conditions will ultimately allow specifically targeted treatments.

2. Models of Motor Neuron Disease

A number of mouse models of lower motor neuron degeneration or neuronopathy are currently available for experimental therapeutics. These include a number of naturally occurring mutant mice: wobbler (wr); progressive motor neuronopathy (pmn); neuromuscular degeneration (nmd); wasted (wst); motor neuron degeneration (mnd and mnd2); motor neuron disease (mneu); and muscle deficient (mdf). In all these mice, the trait is autosomal recessive, reflecting a presumed loss of function of the disease gene. To date, disease genes underlying these spontaneous mutations have not been identified (211). Within the past decade, transgenic mouse models have been created whose features closely model human motor neuron degenerative disease. These include mice with engineered abnormalities of 1 of the neurofilament subunits, and mice overexpressing mutant Cu/Zn superoxide dismutase (SOD1) that has been shown to underlie a subset of familial ALS cases (212–216). In the case of most of the transgenic mouse models, lower motor neuron pathology or degeneration is most likely the result of a gain of function related to the neurofilament or mutant SOD1. The comparable gene knockout strategies result in more subtle pathology (217,218) in the mice.

3. Potential Gene-based Therapies for Motor Neuron Disease

Putative therapeutic approaches for motor neuron disease fall into 4 major categories, based on hypothetical pathogenetic

mechanisms or cell death pathways. Evidence that excess glutamate excitotoxicity can lead to motor neuron death mediated via non-N-methyl-D-aspartate (NMDA) receptors suggests that pharmacological agents targeting the non-NMDA receptors might provide a promising strategy. Motor neuron death has been linked to excitotoxicity involving non-NMDA glutamate receptors (219–221) and related calcium-dependent pathways (222,223) in ALS and in in vitro models. These same phenomena have now been implicated in mutant SOD1-mediated motor neuron death (224,225). Related evidence supports a secondary role for voltage-gated calcium channels and downstream intracellular calcium dysregulation in motor neuron death (225). In an in vitro model, expression of the cytoplasmic calcium-binding protein calbindin-D28K was able to rescue mouse spinal motor neurons from death downstream of mutant SOD1 expression, exogenous glutamate, or paraquat (225). Studies of the efficacy of calbindin or parvalbumin in delaying onset of motor neuron degeneration, or prolonging survival of mutant-SOD1 transgenic mice are currently underway. Some beneficial effects were observed with administration of 2 antiglutamatergic agents, riluzole and gabapentin, to the transgenic mice (226). Riluzole is currently the only medication for which effect on human disease has been demonstrated (227).

Accumulated neuronal damage resulting from oxidative stress over the course of decades may be a common pathogenetic mechanism shared by neurodegenerative disorders, including ALS and PD. Oxidative damage has been documented at the molecular level (228,229). Agents that counteract oxidative stress have had mixed success in preventing motor neuron degeneration in in vitro models (225), transgenic mice, and ALS patients themselves. Some beneficial effects in transgenic mice (either delayed onset, prolonged survival, or improved function) were observed with dietary supplementation with vitamin E (226); a modified catalase, given subcutaneously (230); and treatment with trientine and ascorbate (231). N-acetyl-L-cysteine reduced lower motor neuron degeneration in wobbler (232,233). Nitric oxide synthase inhibitors failed to alter disease course in the SOD1 mutant transgenic mice (234). Collectively, these data suggest enzymes capable of reducing oxidative-free radicals (as described in above neurodegenerative disorders) may be useful in treating motor neuron diseases.

Apoptosis plays a key role in development and morphogenesis. Interplay between antiapoptotic genes such as *bcl-2*, and proapoptotic genes such as *bax* and *bad*, may determine the activation of the enzymatic cascade of caspases leading to condensation and cleavage of nuclear DNA and the hallmark death of the cell with blebbing of the membrane and cytosolic contents. Motor neurons may die by apoptosis (235,236); hence, antiapoptotic strategies have been used in in vitro and transgenic mouse models to prevent motor neuron degeneration. Delayed onset of disease was observed in double transgenic (mutant SOD1 + *bcl-2* overexpression) (237) or in transgenic mice injected intraspinally with a recombinant AAV vector encoding the antiapoptotic gene *bcl-2* (238). Some beneficial effects were also seen with the caspase inhibitor, zVAD-fmk (239). Ultimately, protection against apoptosis cannot prevent motor neuron death (240). The contribution of apoptosis to the etiology of motor neuron death is still being debated (241).

The use of neurotrophic factors has been the most aggressively pursued approach for therapy of the 1990s. Members of the nerve growth factor (NGF) family—NGF itself, NT3, NT4/5-BDNF, CNTF, LIF and GDNF, and IGF-1 (myotrophin)—have all been tested for their efficacy in supporting survival and/or regeneration of motor neurons in in vitro and mouse models. These factors may provide trophic support to the neuronal cell body, promote neurite elongation/sprouting, or reinnervation of the neuromuscular junction. Figure 11 depicts the neuroprotective effect of AAV-GDNF on motor neurons in a mouse model for ALS (242). Ad vectors have been employed to introduce CNTF, BDNF, and NT-3 in rodent models of motor neuron degeneration (234–236). Based on data from the earliest of these studies, CNTF, BDNF, GDNF, and myotrophin have all been used in some stages of clinical trials in ALS patients (243,244). In one approach, microencapsulated cells, genetically engineered to secrete CNTF, were implanted in lumbar intrathecal space (245). Results from that and others clinical trials have been discouraging (246,247). However, a promising gene therapy approach in familial ALS transgenic mice employed intramuscular grafting of myoblasts that were genetically modified to secrete the neurotrophic factor, GDNF, using a hybrid EBV/retroviral vector. The mice demonstrated reduced motor neuron loss and reduced disease progression (248).

Taken together, these studies suggest that the most effective therapies for motor neuron degeneration will include agents drawn from several of the classes described above. Further basic research addressing issues of vector, mode of delivery, and up-regulation and control of expression of the therapeutic agent (e.g., neurotrophic factor) is crucial to the design of improved gene therapy strategies for motor neuron diseases (249–251).

III. FUTURE OF GENE THERAPY AND NEUROLOGICAL DISEASES

The ability to deliver genetic material to cells of a living organism has revolutionized the conduct of biological research and created new approaches in medicine. Although in its infancy, gene therapy offers has potential advantages over traditional pharmacological therapy: a permanent correction of genetic anomaly thereby precluding the need for repetitive dosing. Neurodegenerative disorders affect millions of people, account for billions of dollars in health care costs annually, and as a group of diseases are without available curative therapy. As technology improves, this will lead to a better understanding of the underlying molecular mechanisms that constitute the pathogenesis of neurodegenerative disease. With this, the full potential of gene therapy will be realized.

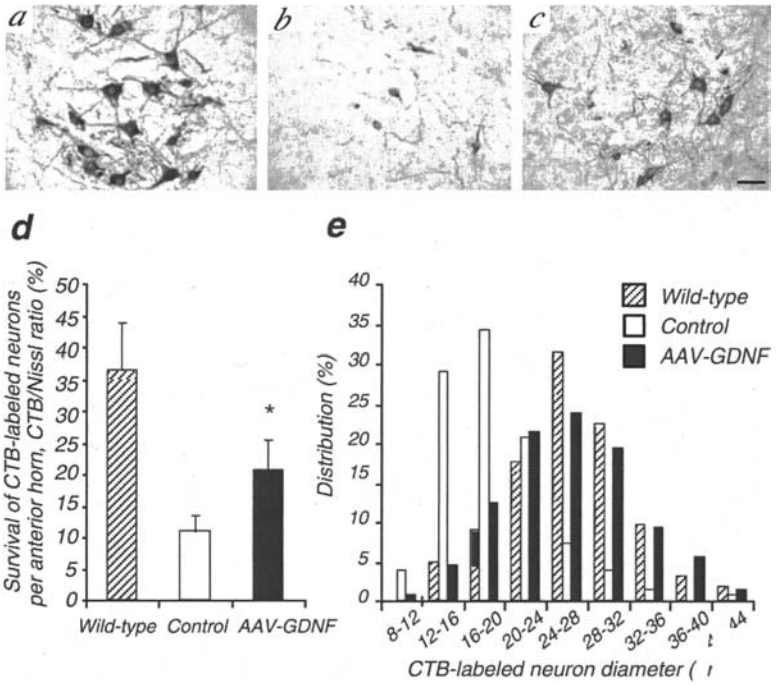

Figure 11 Delivery of AAV-GDNF exhibits neuroprotective effects on motoneurons in a mouse model of amyotrophic lateral sclerosis. Mice bearing the G93A human SOD1 mutation were injected intramuscularly at 9 weeks of age with 3×10^{10} particles of a GDNF-expressing AAV vector (AAV-GDNF) or a β-galactosidase control vector (AAV-LacZ). The illustrated photomicrographs depict the effect of GDNF on motoneurons that retained axonal projections. The neural tracer CTB was used to label motoneurons in the ventral horn at 110 days of age in wild-type (Panel A), AAV-LacZ-injected ALS mice (Panel B), and AAV-GDNF-injected ALS mice (Panel C) following intramuscular injection. AAV-GDNF vector-treated mice exhibited significantly more large CTB-labeled motoneurons than control treated ALS mice (Panel D). The value represents the CTP/Nissl ratio (average number of neurons per anterior horn). The shift in motoneuron size toward a smaller diameter was markedly retarded in AAV-GDNF vector-treated mice compared to AAV-LacZ treatment (Panel E). Scale bar, 50 μm. (From Ref. 242, © 2002 Society for Neuroscience.)

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Principles of Gene Therapy for Inborn Errors of Metabolism

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I. INTRODUCTION

A. Historical Perspective on Inborn Errors of Metabolism

Inborn errors of metabolism (IEM) have played a central role in the formulation of modern genetics. The hallmark of IEM is the accumulation of a biochemical in a bodily tissue. With the development of chemical analytical techniques, it became possible to identify and measure these biochemicals and correlate them with specific diseases (Fig. 1). Knowledge of metabolic pathways enabled enzymatic defects to be identified, which eventually led to discovery of the cognate proteins and genes.

The appreciation that inborn susceptibilities play important roles in diseases was first promulgated at the beginning of the 20th century by Alfred Garrod. He formulated the concept of an inherited metabolic disease on the basis of his studies of patients with alkaptonuria, albinism, cystinuria, and pentosuria. Cognizant of the laws of Mendel, he postulated that the relevant biochemical accumulates due to a metabolic block that is inherited in a recessive process.

The one gene–one enzyme principle developed by Beadle and Tatum provided the next conceptual framework for understanding IEM. This principle provides that metabolic processes are the result of specific enzymatic steps, which are under the control of a single gene. A mutation in a gene leads to deficiency of the enzyme that catalyzes the specific step. The molecular basis for a defective enzyme was provided by Pauling and Ingram's experiments on sickle-cell anemia, while the molecular basis for a defective gene was provided by Watson and Crick and subsequent elaboration of the central dogma. This dogma defines the flow of information as proceeding from DNA to RNA to protein. The objective of gene therapy is to modulate the flow of genetic information so as to attenuate the disease state.

B. Historical Perspective on Gene Therapy of IEM

IEM have also played a central role in the formulation of gene therapy (1). In fact, many of the first human clinical trials in gene therapy were for IEM. In the late 1960s, S. Rogers attempted to treat 3 siblings with arginase deficiency by injecting them with the Shope virus on the basis of the incorrect assumption that the virus contained an arginase gene. While being ahead of his time in anticipating the development of viral vectors, the injections had no effect on the subjects' arginine levels. In the more modern era of gene therapy, the first human trials for treating a disease involved children with severe combined immuno-deficiency (SCID) caused by a deficiency in adenosine deaminase (ADA).

In addition to human gene therapy trials, IEM have played an important role in the development of gene therapy tools (1). Cell lines deficient in hypoxanthine phosphoribosyl transferase (HPRT) and hypoxanthine/aminopterin/thymidine (HAT) selection media (developed by W. and E. Szybalski) enabled the selection for genetically modified cells that take up the HPRT gene in conjunction with other foreign genes. Similarly, cell lines deficient in thymidine kinase (TK) can be used for gene transfer selection.

With the advent of positional cloning and the human genome project, disorders are being linked to defective genes without any understanding of how metabolism has been disrupted. The current challenge will be to identify how the defective gene leads to a disturbance in development or homeostasis. Gene transfer and expression in animals and humans will provide critical tests for hypotheses of pathogenesis.

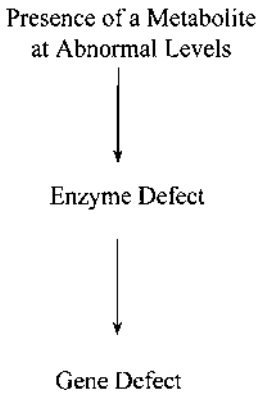

Figure 1 Flow of information in elucidating the genetic basis of metabolic disorders.

II. BASIS FOR GENE THERAPY FOR IEM

A. Types of IEM

One common type of IEM is caused by deficiency of an enzyme that catalyzes the conversion of one chemical to another (Fig. 2a). Deficiency of a specific enzyme can cause disease through 3 separate mechanisms: (1) excessive accumulation of substrate to toxic levels, (2) deficiency of an essential product, or (3) metabolism of the substrate through alternative biochemical pathways leading to toxic secondary metabolites. Examples of such IEM include phenylketonuria and methylmalonic aciduria.

IEM can also be caused by deficiency of protein that is involved in the transport of metabolite (Fig. 2b). Examples include the cystine transporter in cystinosis and the LDL (low-density lipoprotein) receptor in familial hypercholesterolemia.

Other genes relevant to IEM are required for the proper formation of organelles (Fig. 2c). Neonatal adrenoleukodystrophy and Zellweger syndrome are caused by defects in genes that are required for the proper formation of peroxisomes.

B. Different Pathogenesis Models for IEM

The pathogenesis of IEM can be explained by several models (Fig. 3). One major category includes IEM in which organ dysfunction occurs by a circulating toxic metabolite (Fig. 3a). Another major category is organ dysfunction resulting from a cell autonomous process (Fig. 3b). Although these concepts are useful in formulating gene therapy approaches, it should be appreciated that they are only models and that our understanding of the pathogenesis for many IEM is incomplete. In fact, gene therapy trials may provide decisive information concerning the mechanism by which the metabolic defect leads to the diseased state.

C. Circulating Toxic Metabolite

In this class of disorder, a metabolite accumulates in one tissue as result of an enzymatic deficiency (Fig. 3a). This leads to increased metabolite levels in the blood and toxicity in other tissues. The prototype for this type of disorder is phenylketonuria in which deficiency of hepatic phenylalanine hydroxylase leads to increased blood levels of phenylalanine and toxic effects to the developing brain. Familial hypercholesterolemia is another IEM that fits this model. Deficiency of the LDL receptor in the liver leads to increased levels of LDL and subsequent damage to the coronary arteries.

A corollary of this model is intraorgan toxicity from a metabolite that accumulates in an extracellular space within the affected tissue. It is particularly applicable to the central nervous system (CNS). Some IEM associated with neurological dysfunction may be caused by a toxic metabolite that accumulates within the brain and circulates in the cerebral spinal fluid (CSF). Gene therapy could then be predicated on providing gene expression in any cell within the brain as long as the expressed enzyme could lower levels of the toxic metabolite in the CNS. The cerebral spinal fluid could provide the conduit for such exchange.

A metabolic defect in one tissue could also harm another tissue by decreasing the circulating level of a metabolite. For example, a defect in gluconeogenesis that occurs in the liver and muscle (e.g., glycogen storage disorder) can cause hypoglycemia and damage to the brain.

D. Cell Autonomous Toxicity

In other IEM, the metabolic defect only leads to toxicity to the cell that has the metabolic deficiency (Fig. 3b). Cellular toxicity results from either increased or decreased levels of a metabolite within the affected cell. For these disorders, gene therapy would be effective only if the normal gene is targeted to the dysfunctional cell.

E. Method of Gene Correction

A variety of parameters of expression are important determinants of the ability of gene therapy to treat specific disorders. Some generalizations can be made concerning the expression requirements for IEM (Table 1). Most IEM are recessive conditions, and addition of a single gene copy is sufficient to correct the disease phenotype. In effect, gene addition converts the patient to a biochemical state analogous to that of a carrier. For those patients with single-point mutations, targeted gene correction using gene conversion or homologous recombination is a possible therapy, but gene correction is not necessary if a functional gene can be added. The obvious therapeutic gene to be added in IEM is the human gene that is defective in the disease state, but it is conceivable that a therapeutic effect could be achieved using another gene. For example, a gene from another species could metabolize a toxic metabolite by a different mechanism.

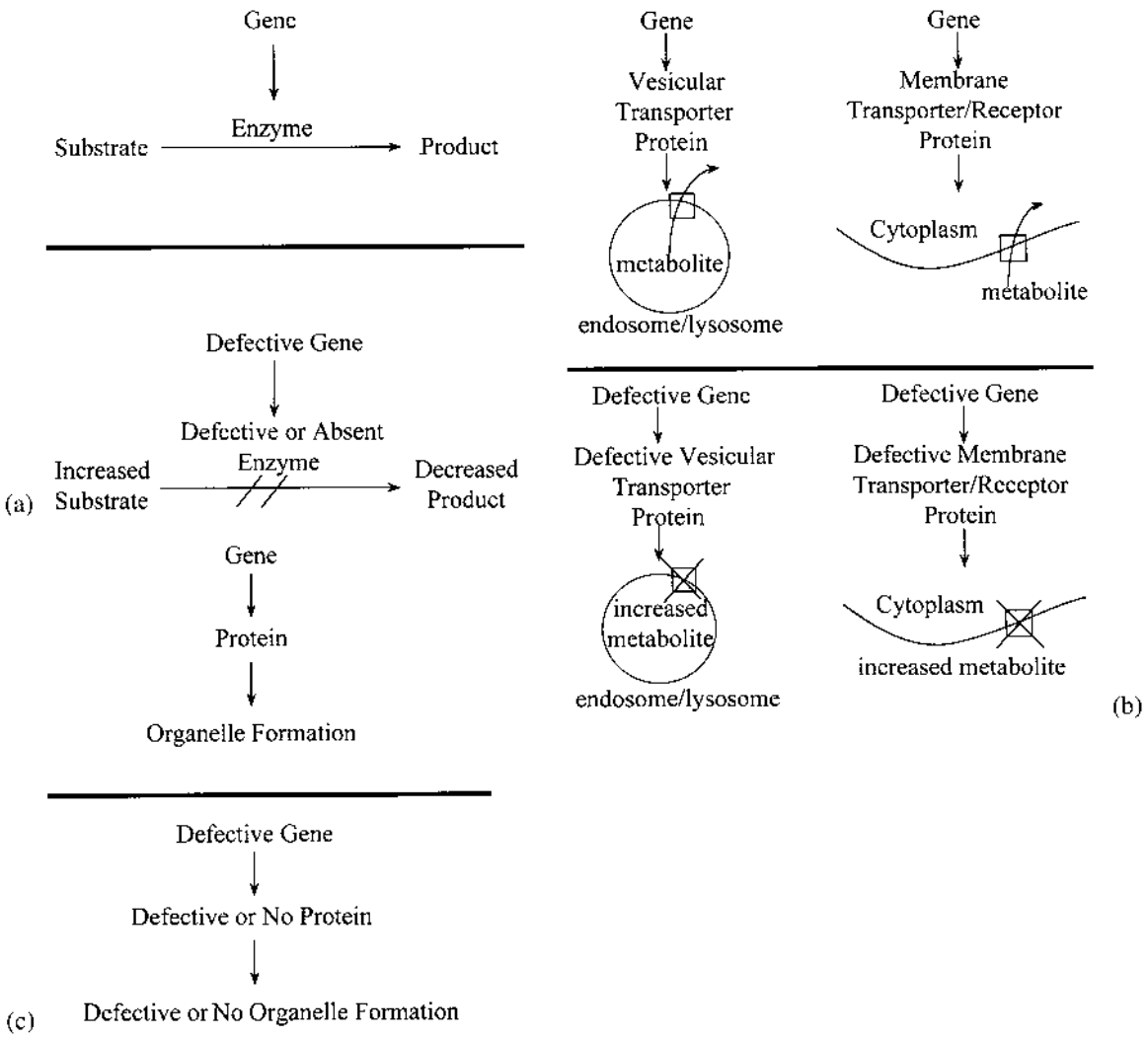

Figure 2 The types of normal functions that are disrupted in IEMs: (a) defects in enzymatic activity, (b) defects in the uptake or transport of metabolites, and (c) defects in the formation of organelles.

F. Requirements for Expression Persistence

For most IEM, gene expression does not have to be regulated and can be constant. Most genes involved in IEM are considered ''housekeeping'' genes. In contrast, in diabetes mellitus, insulin expression has to be regulated in response to blood glucose levels.

Given that IEM are chronic conditions, persistent expression is needed. It would be best if gene correction and therefore a ''cure'' could be done with 1 or few administrations. If expression cannot be persistent after 1 gene dose, then repetitive administrations are required. Repetitive administrations can be problematic for some vectors such as adenoviral vectors that induce neutralizing antibodies. Loss of expression from vectors can be a result of removal of the foreign DNA, promoter suppression, or rejection of the foreign gene product.

Immune effects can arise even if the gene product is intracellular because all parts of proteins are presented to the immune system via the MHC I complex. The important issue is whether, in the disease state, the patient expresses any residual native protein and is immunologically tolerant to the normal gene product. One measure of this is whether tissues from the patient exhibit cross-reactive material (CRM), protein that cross-reacts with antibodies against the native protein. This is best determined by performing immunoblot (Western blot) analysis. Even if protein is not present, native protein could have been produced but be unstable. Expression of the foreign gene in such a patient may not induce an immune effect because the protein is not recognized as foreign. Further experi-

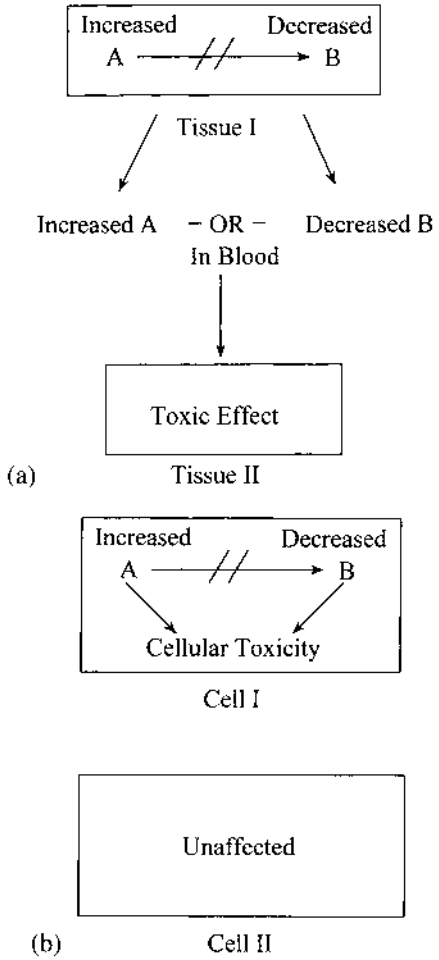

Figure 3 Two models by which IEM leads to cellular toxicity: (a) circulating toxic metabolite, and (b) cell autonomous toxicity.

Table 1 Generalizations Concerning Expression Requirements for Gene Therapy of IEM

Expression parameter	Requirement
Method of modification	Gene addition is sufficient
Therapeutic gene	Normal gene that is defective in patient
Duration	Persistent
Regulation	Not needed
Levels	>5% of normal levels
Target tissues	Liver, CNS, blood cells, muscle, heterologous expression possible in some disorders

ence is necessary to determine whether the immune system will prevent stable expression of the normal gene in patients with IEM.

G. Requirements for Expression Levels

The level of expression is a critical determinant for the success of a gene therapy. For most IEM, foreign gene expression only has to be greater than 5% of normal levels in order to attenuate the majority of the diseased state. This is based on clinical experience in which the percent of residual enzyme activity is correlated with the phenotype. In many IEM, people with more than 5% of normal enzymatic activity are free of symptoms. If enzymatic activity is between 1% and 5%, their clinical course is less severe than patients with 0% of enzymatic activity.

Although the total enzymatic activity is one measure, the percent of cells expressing the foreign gene may also be important. Overexpression in a few cells may not lead to a therapeutic effect if the expressed enzyme alone cannot completely produce the metabolic conversion. The protein deficient in the patient may be part of an enzymatic complex so that overexpression of 1 component would not necessarily lead to higher activity of the complete complex. Similarly, other enzymatic steps, cofactors, or transport of metabolites may limit the ability of the cell to perform the required metabolic conversion at a rate higher than the normal level. If so, the therapeutic gene has to be expressed in more than 5% of the target cells.

H. Target Tissues

Details of the pathogenesis for the IEM need to be understood, and the target tissue has to be tailored for each disorder. For IEM that fit the ''circulating toxic metabolite'' model, the therapeutic gene does not necessarily have to be targeted to the tissue that normally expresses the affected gene (Fig. 4). Although correction of the deficient enzymatic activity in the affected organ would be most straightforward, expression within a heterologous tissue (different from that which normally expresses the enzyme) could clear the circulating toxic metabolite and attenuate the disease state. For this approach to be effective, the enzyme must be functional within the heterologous tissue. Restrictions on enzymatic function can include requirements for protein subunits, cofactors, substrate, and clearance of product. Given the ability for several gene transfer systems (e.g., plasmid DNA, adenoviral vectors, and AAV vectors) to express foreign genes stably in muscle, it will be a useful tissue for many heterologous gene therapy approaches. Blood cells derived from genetically modified stem cells are another candidate tissue for heterologous gene expression if the problems associated with stable foreign expression are solved.

For IEM that fit the ''cell autonomous'' model, expression within the affected cell is generally required. The exception is for the lysosomal storage disorders in which the enzyme can be transferred from one cell to another.

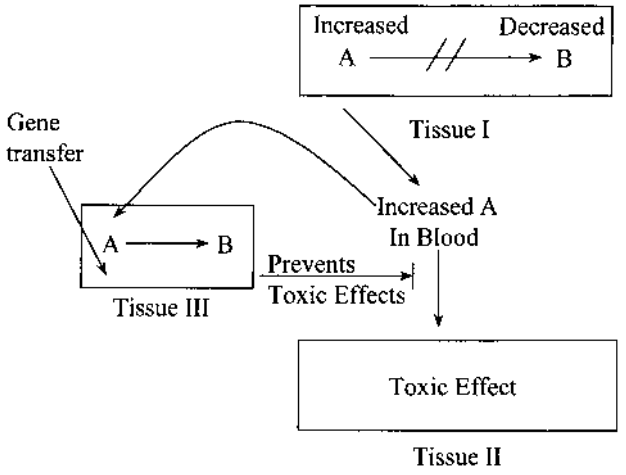

Figure 4 Heterologous tissue expression of a therapeutic gene to treat an IEM in which a circulating toxic metabolite causes the diseased state.

For IEM that affect the brain, ''global'' gene expression throughout the brain may be required. Alternatively, specific neurological symptoms could be treated by targeting specific regions of the brain. For example, in Lesch–Nyhan syndrome, choreathetoid movements could be treated by targeting the basal ganglia.

I. Mitochondrial Disorders of Oxidative Phosphorylation

Several IEM are caused by defective oxidative phosphorylation within the respiratory chain complex of mitochondria. A unique feature of mitochondria is that 13 of the more than 80 respiratory chain subunits are encoded within the mitochondrial genome. The mitochondria also contain 22 transfer RNA (tRNA) and 2 ribosomal RNA that enable protein synthesis within the mitochondria. The remaining 70 or so respiratory chain subunits are encoded within the nuclear genome. These proteins are produced within the cytoplasm and contain an amino terminus that targets their entry into the mitochondria by interacting with a number of chaperone and transport proteins.

For disorders caused by mutations in the nuclear encoded respiratory chain genes, the gene therapy approaches described above are germane. However, disorders caused by mutations in the mitochondrial genome offer additional challenges for gene therapy. One approach would be to express the deficient subunit within the nucleus, regardless of its native mitochondrial origin. The subunit could be modified to contain an amino leader sequence to enable entry into the mitochondria.

The other approach of genetically modifying the mitochondrial genome is at an early conceptual stage. Toward this end, a peptide mitochondria-targeting sequence has been covalently attached to oligonucleotide to enable mitochondrial entry. The oligonucleotide could correct a point mutation by some type of gene conversion or recombination process. Point mutations occur in mitochondrial disorders such as Leber hereditary optic atrophy (LHON), myoclonic epilepsy and ragged-red fiber disease (MERRF), and mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS). An alternative treatment approach would be the addition of functional tRNA genes to patients with mitochondrial disorders such as MERRF or MELAS that are caused by tRNA mutations. The treatment of mitochondrial DNA deletion diseases would require the delivery of larger DNA sequences (>5 kb), which would be more challenging. Deletions occur in disorders such as Kearns–Sayre syndrome. Another option would be to deliver normal mitochondria en toto.

Different mitochondria can proliferate in a tissue at different rates. This may explain why inborn and somatic (acquired) mitochondrial defects often present in later life. Any genetic modification of mitochondria must enable the corrected mitochondria to have a proliferation advantage over the abnormal mitochondria in order to achieve a permanent cure. A final challenge for mitochondrial disorders is that they often involve the nervous system, which is less accessible than other organs to therapeutic endeavors.

J. Newborn and Prenatal Screening

Gene therapy for IEM will have a significant impact on newborn screening programs and vice versa. Screening for IEM at birth enables gene therapy to be initiated prior to the onset of symptoms and any irreversible tissue damage and thereby increases the value of the gene therapy. Irreversible brain damage occurs in many IEM when a neonatal metabolic crisis is not prevented. For example, the extent of perinatal hyperammonemia in a urea cycle defect, ornithine transcarbomylase deficiency (OTC), has been directly correlated with intelligence in later life.

One criterion for the initiation of newborn screening for a particular disorder is whether an effective treatment exists. The development of effective gene therapy for a disorder could satisfy this criterion. Another criterion is the availability of a reliable, inexpensive laboratory method for disease detection.

Currently, most states in the United States and many other nations are screening for phenylketonuria and galactosemia. Screening for maple syrup urine disease or homocysteinemia is less common. Tandem mass spectroscopy procedures are being developed for analyzing blood spots in amino acids and organic acids conjugated to carnitine (acylcarnitines) in order to detect many of the disorders in amino acid and fat metabolism and organic acidurias. Such comprehensive newborn screening programs developed in conjunction with new gene therapies will have a major impact on the morbidity and mortality of IEM.

Many IEM can be reliably diagnosed in the prenatal period. As intrauterine gene therapy approaches are developed, IEM will be good candidates for such approaches. One potential

advantage of prenatal approaches may be a decreased chance of an immune recognition of the therapeutic gene product.

III. GENE THERAPY OF SPECIFIC DISORDERS

A comprehensive review of the tremendous progress in the gene therapy for IEM is beyond the scope of this review. In fact, gene therapy studies have been conducted in almost every type of IEM. Instead, specific IEM were chosen either because they illustrate the above principles or for their important historical role.

A. Aminoacidopathies

The aminoacidopathies are a heterogeneous group of recessively inherited enzyme deficiencies that are associated with the accumulation of specific amino acids in blood and other tissues. The best known and most studied aminoacidopathy is phenylketonuria (PKU) (Table 2) caused by deficiency of the liver enzyme phenylalanine hydroxylase (PAH) (Fig. 5). PAH deficiency prevents the hydroxylation of phenylalanine to tyrosine and leads to excessive accumulation of phenylalanine in the body. If PKU is left untreated in an infant, poor brain and physical growth, seizures, and mental retardation will result from increased levels of the circulating toxic metabolite phenylalanine. Other examples of aminoacidopathies include tyrosinemia, maple syrup urine disease, homocystinuria, and ornithine transcarbamoylase (OTC) deficiency (Table 2). Each of these enzyme deficiencies leads to the accumulation of a different specific substrate and causes a different symptom complex.

Contemporary therapy for these diseases is based on an understanding of the pathogenesis involved in each case, and

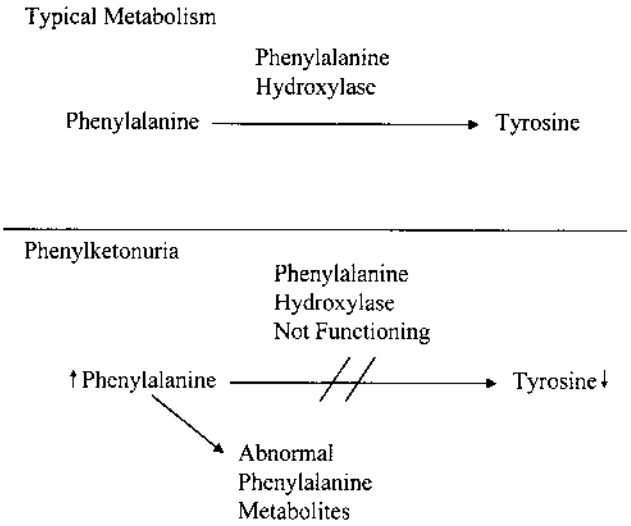

Figure 5 Pathogenesis of phenylketonuria.

the design of any gene therapy protocol must also be grounded upon a rational understanding of the specific disease pathophysiology. For example, high levels of phenylalanine in PKU are toxic to the developing brain, and reducing blood phenylalanine levels is critical to successful treatment of PKU. However, some symptoms of PKU may be caused by deficiencies of specific neurotransmitters, such as dopamine, that are synthesized from tyrosine. In tyrosinemia, elevated tyrosine levels do not appear to be directly toxic, but production of the toxic metabolite succinylacetone through an alternative biochemical pathway causes severe liver damage. So, for PKU, a successful therapy will both remove phenylalanine and restore tyrosine levels, while removal of tyrosine from individuals with tyrosinemia is less important than stopping the production of succinylacetone. These considerations must play a role in the design of any gene therapy protocol.

In many aminoacidopathies, the deficient enzyme is normally either exclusively or primarily expressed in liver; liver is the obvious target for gene transfer in these diseases (2). However, for select disorders, circulating toxic metabolites may be effectively removed from the body by enzyme expressed in a tissue other than liver. The concept of expressing in an alternative tissue a protein that is normally restricted to a specific organ is known as heterologous gene therapy. As an example, PAH expression in skeletal muscle, if supplied with the necessary cofactors, might effectively clear phenylalanine from the circulation of a person with PKU. Gene targeting to the liver may not be essential for some aminoacidopathies. For other diseases, specific pathophysiological features limit the effectiveness of a heterologous gene therapy approach. In OTC deficiency, the substrate for OTC, carbamyl phosphate, is produced only locally in the liver and does not appear in the circulation. In tyrosinemia, liver damage is mediated by local production of succinylacetone in hepatocytes; removal of succinylacetone from the circulation without preventing its production in the liver might not alter the course of tyrosinemia. If possible, disease-specific pathophysiological features and the effectiveness of any gene therapy approach should be demonstrated in an animal model of the disease, if one is available, prior to application of the method in humans. Table 2 lists animal models available for the study of some aminoacidopathies.

Gene transfer experiments to treat PKU illustrate the difficulties and complexities of gene therapy for aminoacidopathies and other liver diseases (2). The availability of a mouse model, the *Pah*^{enu2} mouse, that accurately portrays human PAH deficiency has allowed significant advances in PKU gene therapy research. Soon after cloning the PAH gene, Dr. Savio Woo and colleagues pioneered gene transfer into liver and demonstrated that PAH activity could be expressed in cultured fibroblasts or PAH-deficient hepatocytes from a PAH cDNA. This was accomplished using a variety of gene transfer vectors, including recombinant retrovirus. Scaling up to perform retroviral-mediated liver-directed gene therapy in a whole animal proved more problematic. Integration of the retroviral DNA into the target genome requires mitotic division of the target cell; the mitotic rate of hepatocytes in vivo

Table 2 Summary of Select Aminoacidopathies

Disease	Deficient enzyme	Elevated blood amino acids	Animal model?
Phenylketonuria	Phenylalanine hydroxylase (PAH)	Phenylalanine	*Pah*^{enn2} mouse
Tyrosinemia type I	Fumarylacetoacetate hydrolase (FAH)	Tyrosine	FAH knockout mouse
Ornithine transcarbamoylase deficiency	Ornithine transcarbamoylase (OTC)	Glutamine; elevated blood ammonia	Sparse fur (*spf*) mouse
Maple syrup urine disease (MSUD)	Branched-chain keto acid dehydrogenase	Leucine, valine, isoleucine	Hereford inbred calf MSUD
Homocystinuria	Cystathionine β-synthase	Homocystine	None

is estimated to be only about 1% per year in humans. So, retroviral-mediated liver-directed gene therapy requires an ex vivo approach in which part of the liver is surgically removed from an animal, the hepatocytes are cultured, allowed to divide, transfected with the recombinant retrovirus, and then the treated hepatocytes are infused back into the animal via the portal venous system. Using this approach and the β-galactosidase reporter gene, Woo and colleagues demonstrated that approximately 1% of the hepatocytes in the liver of a dog could be induced to permanently express the reporter gene. Unfortunately, successful treatment of PKU (and probably other aminoacidopathies) requires enzymatic reconstitution of at least 5% to 10% of the liver. Successful retroviral-mediated ex vivo gene therapy in the PKU mouse has not been reported. This approach has, however, been employed successfully in the treatment of murine tyrosinemia type I (3). In this mouse model, enzymatically corrected hepatocytes have a survival advantage over enzyme-deficient cells in the host; although they initially constitute only a very small fraction of the liver, retrovirus-treated enzyme-expressing hepatocytes gradually repopulate the entire liver. Recombinant retroviral-mediated gene therapy may be useful for disorders that require correction of only 1% to 2% of the liver to effect a phenotypic change or in situations where corrected cells have a competitive advantage over the native hepatocytes.

Recombinant adenoviral vectors are another promising gene delivery vehicle for in vivo application. In contrast to retroviral vectors, recombinant adenovirus can be produced and purified to higher titers and are capable of efficiently infecting nondividing cells. A recombinant adenovirus containing the PAH cDNA has been infused into the portal circulation of PKU mice (4). In this experiment, hepatic PAH activity was reconstituted to 5% to 20% of control levels. Complete normalization of plasma phenylalanine levels occurred in animals with hepatic PAH activity equivalent to at least 10% of that in control animals. However, the effect had disappeared by 3 weeks following the treatment, and no hepatic PAH activity was detected following a second treatment with the adenoviral vector. The stability of expression from adenoviral vectors administered in vivo is limited by the immune response of the host against the vector or the reporter gene product. In experimental trials of adenovirus-mediated, liver-directed gene therapy employing a variety of different therapeutic genes to treat several different animal models, gene expression has been stable for 7 to 10 days and then has decreased to undetectable levels over the next few weeks.

Further alteration of the adenoviral genome has resulted in recombinant adenovirus that is less immunogenic than first-generation adenovirus vectors. Liver OTC deficiency in the sparse fur mouse was corrected for at least 2 months following infusion of a second-generation recombinant adenovirus containing the human OTC cDNA (5). This vector is currently being used in a human clinical trial involving adult females with partial OTC deficiency. Further efforts to develop even more effective adenoviral vectors continues.

The search for the ideal liver-directed gene-transfer vector continues. Along with further modification of recombinant adenovirus vectors, the development of new viral and nonviral vectors targeted to liver has expanded the therapeutic armamentarium of the gene therapist. Early animal trials with recombinant adeno-associated virus (AAV) and lentivirus vectors have shown substantially better persistence of gene expression than with recombinant adenovirus and much higher levels of gene product than with recombinant retrovirus. Nonviral methods such as infusion of naked plasmid DNA under physical and osmotic pressure directly into the venous circulation of liver or the bile duct aim to altogether avoid difficulties with immunogenic effects of a viral delivery system. Although much further research in animal models is needed before human clinical trials should be attempted, several new gene delivery systems demonstrate the promise of physiologically significant stable gene expression in liver.

B. Organic Acidurias

Propionic aciduria and methylmalonic aciduria are 2 organic acidurias that have been well studied and are excellent candidates for gene therapy (Fig. 6). In severe cases, patients present in the neonatal period with coma, metabolic ketoacidosis, and hyperammonemia. With vigorous medical support, they can survive this initial metabolic crisis, but they then must adhere to a strict diet restricted in protein intake. Despite di-

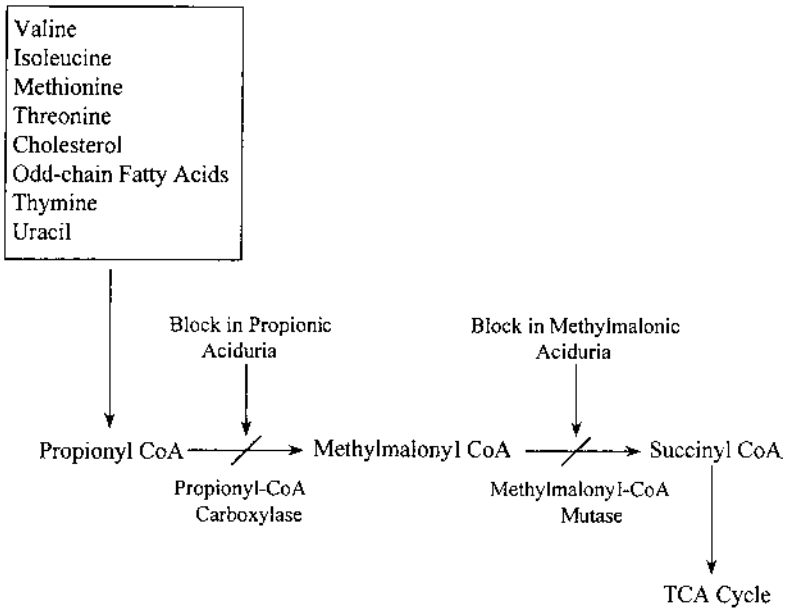

Figure 6 Enzymatic deficiencies in methylmalonic aciduria or propionic aciduria. TCA, tricarboxylic acid cycle.

etary therapy, they continue to have metabolic crises that can be life-threatening. Given the inadequacy of dietary therapy, a gene therapy approach is needed.

Both disorders are caused by enzyme deficiencies in the metabolism of 3-carbon species that are generated from the catabolism of amino acids and other metabolites (Fig. 6). Methylmalonic aciduria is caused by a deficiency of methylmalonyl-CoA carboxylase activity that is a result of a defect either in the apoenzyme or in the active form of vitamin B_{12}. Patients with the latter defect often respond well to treatment with large amounts of vitamin B_{12} and are therefore in less need of gene therapy.

The prominent target tissue for both disorders is presumed to be the liver. Hepatorenal transplantation has been successfully employed in a patient with a severe form of methylmalonic aciduria. Nonetheless, the major pathology associated with these organic acidurias is due to circulating toxic metabolites. The associated enzymes are normally expressed in many tissues including leukocytes, muscle, and fibroblasts. Therefore, these heterologous tissues should be explored in gene therapy preclinical studies. Unfortunately, animal models for these disorders do not exist at the present time.

Five percent of normal enzymatic activity in either propionic aciduria or methylmalonic aciduria is associated with a benign clinical course, indicating that this level of expression in a gene therapy should be sufficient to realize a large clinical benefit. This level of expression may have to be distributed over approximately 5% of the cells because overexpression of the relevant genes may not lead to a proportional increase in metabolic flux through the 3-carbon pathway. For example, in propionic aciduria, the propionyl-CoA carboxylase has 2 different subunits, α and β. In patients with a defect in the β subunit, overexpression would require gene transfer with both subunits. However, in patients with a defect in the α subunit, overexpression of the α subunit may be sufficient because the β subunit is produced in a 5-fold excess over that of the α subunit.

C. Lysosomal Storage Diseases

The common feature of lysosomal storage diseases is the inappropriate accumulation of normal cellular components within lysosomes. This storage of material is visible in cells by light microscopy as very large lysosomes that displace a large part of the cytoplasm. This class of disorders is caused by deficiency of specific lysosomal enzymes that are required for the degradation and recycling of glycoproteins and other cellular components. Without a specific degradative enzyme, the substrate for the reaction accumulates and cannot be removed from the lysosome. The clinical phenotype of each disease is dependent on the tissue type most affected by storage and by the accumulation rate. Physical findings that are suggestive of lysosomal storage include enlargement of the liver and spleen, anemia and thrombocytopenia due to replacement of normal bone marrow by stored material, destruction of bone, and for those enzymatic deficiencies that cause lysosomal storage in neurons, severe developmental regression, seizures, and other neurological symptoms. Not all of these problems are present in all lysosomal storage diseases; each different enzymatic deficiency presents with a specific phenotypic complex.

The challenges of gene therapy for lysosomal storage diseases are illustrated by the results of contemporary treatment

with enzyme-replacement therapy or bone marrow transplantation. A major challenge to treating a lysosomal storage disease with gene therapy (in contrast to treatment of a liver enzymopathy) is the necessity of reversing lysosomal storage in multiple separate tissues. No currently available gene transfer technique is capable of delivering DNA to multiple target tissues efficiently. However, many lines of evidence demonstrate that lysosomal enzyme proteins can be produced in isolated tissues or even purified ex vivo and effectively delivered to most target tissues. For example, glucocerebrosidase, the enzyme deficient in Gaucher's disease can be produced in vitro using standard recombinant techniques, chemically modified to facilitate lysosomal targeting, and delivered to affected organs by simple intravenous infusion. This therapy if repeated periodically dramatically reduces liver and spleen size, corrects anemia and thrombocytopenia, and possibly prevents bone deterioration, all major debilitating features of Gaucher's disease. So, at least for gene therapy of Gaucher's disease, the enzyme would not need to be locally produced in all affected tissues. The enzyme could potentially be produced in a single target tissue, secreted into the circulation, and taken up by other diseased tissues. The major limitation of this approach is the difficulty of engineering a secreted form of the enzyme that would be efficiently taken up by other cells and incorporated into lysosomes.

Alternatively, the enzyme could be transferred from the site of production to diseased tissues via circulating blood cells. Seminal experiments demonstrated that functional lysosomal enzymes may be transferred directly from a normal cell to an enzyme-deficient cell in tissue culture. Bone marrow transplantation in the treatment of lysosomal storage diseases exploits this phenomenon. Replacement of enzyme-deficient host bone marrow with enzyme-sufficient donor bone marrow yields a population of circulating blood cells of the reticuloendothelial lineage that infiltrates tissues and transfers lysosomal enzyme to the native cells. Bone marrow transplantation has been employed successfully in Gaucher's disease and in select other storage diseases that do not exhibit brain involvement. Apparently either insufficient numbers of corrected cells penetrate the central nervous system or insufficient enzyme is transferred to neurons to successfully ameliorate the neurological phenotype of many lysosomal storage disorders. Presumably, difficulties with correcting enzyme deficiency in the brain will also be a major obstacle to successful gene therapy.

Gene therapy for lysosomal storage diseases has to date focused on gene transfer into bone marrow stem cells for the purpose of supplying enzyme via circulating reticuloendothelial cells (6). Enzymatic correction of Gaucher bone marrow cells in culture has been accomplished with recombinant retroviral vectors. Similar experiments using other lysosomal enzymes in both cultured bone marrow and fibroblasts have been successful. Persistent production of enzyme in circulating blood cells has been demonstrated in rodents. Phenotypic improvement following retroviral-mediated gene transfer into bone marrow has been shown in gus^{mps}/gus^{mps} mice, a β-glucuronidase-deficient mouse model of human mucopolysaccharidosis type VII. As expected, enzymatic correction of bone marrow resulted in amelioration of the somatic symptoms but did not arrest progressive neurological deterioration in this model. However, lysosomal storage in the brain did decrease in mice that had received intracerebral β-glucuronidase-expressing fibroblast implants. Clinical trials of retroviral-mediated bone marrow stem cell-directed gene therapy are underway in humans with Gaucher's disease and in patients with Hunter's syndrome (mucopolysaccharidosis type II) who have little central nervous system involvement.

D. Lesch–Nyhan Syndrome

This X-linked syndrome is caused by a deficiency in hypoxanthine phosphoribosyl transferase (HPRT), an enzyme required for salvaging purines (Fig. 7). It is characterized clinically by increased blood and urine uric acid, mental retardation, choreoathetoid movements, and, most extraordinarily, self-mutilation. It is not understood how a deficiency in HPRT leads to these remarkable neurological sequelae. A genetic mouse model completely lacking HPRT activity does not exhibit any neurological dysfunction except when stressed with amphetamine administration or inhibition of adenine phosphoribosyl transferase (APRT) with 9-ethyladenine. The choreoathetoid movement disorder, however, is postulated to be due to dysfunction within the basal ganglion secondary to disturbed dopamine metabolism.

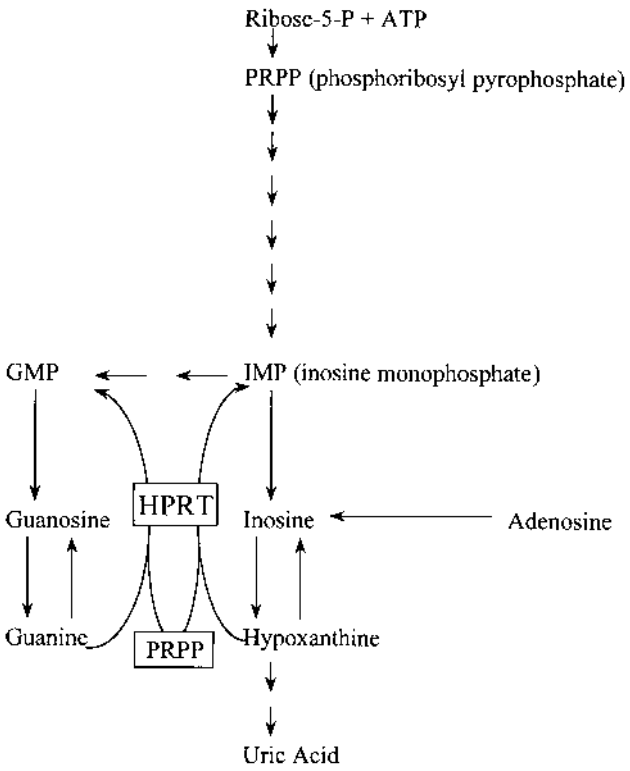

Figure 7 Enzymatic deficiency in Lesch–Nyhan syndrome.

Although the hyperuric acidemia and its sequela can be controlled with allopurinol, the absence of treatment for the neurological symptoms has prompted the search for gene therapy approaches. Historically, Lesch–Nyhan syndrome has played an important role in the development of gene therapy. One of the first demonstrations of the ability of retroviral vectors to correct a genetic mutation was done using the human HPRT gene. The first animal experiment in which a foreign gene was expressed in the brain was done by intracerebrally transplanting fibroblasts genetically modified to express the human HPRT (7). Although HPRT is expressed in all cells, its high levels in the basal ganglia suggest that this area of the brain should be targeted for gene transfer. Prevention of the mental retardation may require more global expression within the brain.

The amount of normal gene expression required to effect relief can be extrapolated from clinical experience. Although it was previously believed that the severity of the syndrome was not correlated with residual enzymatic activity, it is now realized that its severity does correlate with the amount of HPRT activity in whole cells. Patients with 1.6% to 8% of normal activity had choreoathetosis but not mental retardation or self-mutilation.

In summary, this syndrome is an example of a genetic disorder in which therapy is lacking even when so much is known about its genetic and molecular basis. The development of effective gene transfer methods into the brain may not only provide a therapy but will be quite revealing about its pathogenesis.

E. Familial Hypercholesterolemia

Gene therapy has the potential to significantly improve the clinical status of patients with familial hypercholesterolemia (FH), which is caused by a defect in the LDL receptor (LDLR) (Fig. 8). Deficiency in this receptor leads to reduced clearance of LDL by the liver and higher blood levels of LDL. In addition, affected individuals synthesize more cholesterol because the inhibitory effect of LDL on cholesterol synthesis is lost. This inhibition results from decreased HMG CoA reductase activity, the rate-limiting step in cholesterol synthesis.

Heterozygotes with LDLR deficiency occur at a frequency of 1:500 (as common as insulin-dependent diabetes mellitus), making it one of the most common genetic disorders in the United States, Europe, and Japan. Such patients have a 2-fold elevation in plasma cholesterol levels (300–600 mg/dL) and may develop coronary artery disease by the fourth decade of life. Three to 6% of survivors of myocardial infarctions are heterozygotes for FH.

Homozygotes with LDLR deficiency occur much more infrequently (1 in a million), but have much higher cholesterol levels (600–1000 mg/dL) and invariably die from coronary artery disease in their 20s. The severity of the sequelae is attenuated in the homozygotes by a few percent residual LDLR activity. Deaths were much less frequent in those homozygotes who had at least 10% of normal LDLR activity. This indicates that clinical benefit could be achieved by a gene

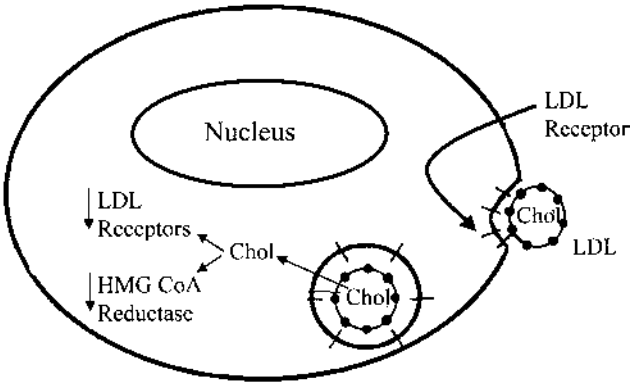

Figure 8 Pathogenesis of familial hypercholesterolemia. Chol, cholesterol; HMG, 3-hydroxymethylglutary; LDL, low-density lipoprotein.

therapy in which only a small percentage of LDLR activity is restored. Furthermore, the severity of this disorder increases the benefit-to-risk ratio of clinical trials and thereby facilitates them. A gene therapy protocol can be first tested in the homozygotes (aided by Orphan Drug Status) and then extended to the more common heterozygotes.

Liver transplantation in children has proven that correction of the LDLR defect in the liver can normalize cholesterol levels. For this reason, gene therapy techniques for FH have been directed at the hepatocyte. Based on preclinical studies in mouse and rabbit LDLR-deficient models, ex vivo gene therapy in 5 homozygous FH patients using retrovirus-mediated LDL receptor gene transfer was performed. This technically challenging protocol yielded a highly variable metabolic response with some improvement in only 1 of the patients (8). This study indicates that important modifications must be made to the ex vivo gene transfer method before gene therapy can be used as a general therapeutic procedure for such patients (9).

Given the borderline results of the human clinical trial, efforts were initiated with adenoviral vectors carrying the LDLR gene. In vivo adenovirus-mediated transfer of the LDLR was shown to be highly effective in reversing the hypercholesterolemia in LDLR knockout mice and WHHL rabbits (10). The important limitation of adenoviral-mediated gene transfer remains the transient expression in vivo after infection of somatic cells with recombinant adenovirus. Nonetheless, these studies demonstrate the proof of principle for the gene therapy of FH by the transfer of the normal LDLR gene and highlight the inadequacies of current gene transfer methods.

Current therapy for hypercholesterolemia (not limited to homozygotic FH) includes the use of HMG-CoA reductase inhibitors, which work by secondarily inducing expression of the LDLR, thereby lowering plasma LDL levels. These agents not only lower serum cholesterol, but also lower all-cause

mortality by at least 30% in men and women who have coronary disease and total cholesterol levels of 215 to 300 mg/dL. However, 2% of patients suffer liver toxicity and 0.2% develop muscle disease requiring cessation of drug administration. These drugs have to be taken once or twice every day for extended periods of time, and compliance is often difficult. A gene therapeutic agent that is administered less than every month (even by intravenous injection) would offer substantial benefit to the patient.

At high efficiencies of liver gene transfer, LDLR gene transfer into the liver could be used to prevent coronary artery disease in the general population. Taking into account all types of hypercholesterolemias, the third National Health and Nutrition Examination Survey (NHANES III) concluded that lipid-lowering therapy was required for 29% of Americans over 20 years of age (11). The Cholesterol and Recurrent Events study showed that patients with coronary artery disease but having ''normal'' LDL cholesterol levels benefited from treatment with a single statin therapy (12). The positive correlation between LDL levels and coronary artery disease is a continuum. In addition, overexpression of the normal LDL receptor in the liver of transgenic mice (4 to 5 times that of the endogenous receptor) prevented diet-induced hypercholesterolemia, suggesting that unregulated overexpression of the LDLR by liver gene therapy would be therapeutic in humans with hypercholesterolemia of various causes (13).

Many individuals develop coronary artery disease from other causes not amenable to statin therapy but that are potentially treatable by gene therapy. Liver gene therapy using the apoB mRNA editing enzyme (Apobec 1) or the VLDL receptor genes could modify LDL cholesterol levels. Other lipoprotein factors besides LDL cholesterol levels influence the onset of coronary artery disease and are amenable to modulation by liver gene transfer. Additional expression of apoA-I in the liver by foreign gene transfer could raise high-density lipoprotein levels and prevent atherosclerosis, as has been demonstrated in mouse and rabbit models. Hypertension, a predisposing factor for coronary artery disease, could be treated by delivering the kalikrein gene to the liver.

IV. SUMMARY

The foundation for treating many IEM by gene therapy has been established. It is clear that the expression of the cognate gene can correct the metabolic disturbance and the disease state in most IEM. As in other types of disorders, clinical success has been thwarted by inefficiencies in the gene delivery and expression systems. As new vectors and expression systems are developed and improved over the next few years, it is anticipated that clinical efficacy will be demonstrated for an increasing number of IEM.

ACKNOWLEDGMENT

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31

Gene Therapy and HIV-1 Infection

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I. INTRODUCTION

Since the discovery that acquired immunodeficiency syndrome (AIDS) is caused by a retrovirus, termed human immunodeficiency virus type I (HIV-1), enormous efforts have been undertaken to develop new pharmaceutical agents to control the spread of this epidemic disease. Such conventional drugs are specifically designed to block the action of HIV-1-specific enzymes, such as the reverse transcriptase or the protease. However, as a result of the high mutation rate of the virus, new virus variants continuously emerge that are resistant to such conventional therapies. Thus, great efforts are currently being made in many laboratories worldwide to develop alternative genetic approaches to inhibit the replication of this virus. With growing insight into the mechanism and regulation of HIV-1 replication, genetic antivirals have been developed that attack basically every step in the viral life cycle. Tissue culture cells have been transduced with genes encoding for such antivirals, and it has been shown that such transduced cells can become rather resistant to HIV-1 infection. However, although such antivirals have been proven to be very effective in vitro, their beneficiary effect in vivo is difficult to evaluate and still remains to be shown. Furthermore, the delivery of genes encoding for such genetic antivirals still constitutes one of the main problems because no efficient gene delivery tools are available at this point, which would enable the robust delivery to the actual target cell in vivo. This chapter summarizes the experimental approaches and current gene delivery techniques to inhibit HIV-1 infection.

II. HIV-1 INFECTION AND CONVENTIONAL PHARMACEUTICAL AGENTS

AIDS is caused by HIV-1. This retrovirus primarily infects and destroys cells of the human immune system, in particular, $CD4^+$ T cells and macrophages. The destruction of such cells leads to a severe immunodeficiency (e.g., the inability to fight other infectious agents or tumor cells). Thus, AIDS patients usually die from secondary infections (e.g., tuberculosis, pneumonia) or cancer (e.g., Kaposi's sarcoma). Enormous efforts have been made to study the life cycle and pathogenesis of HIV-1 in order to find potential targets to block the replication of this virus (Fig. 1). A list of potential targets and appropriate control strategies are described in Figure 2. Some viral proteins, such as the protease, reverse transcriptase, and envelope have been crystallized and their 3-dimensional structures revealed (1–6). These studies were performed to design specific compounds, which would irreversibly bind to the active sites of such enzymes, and therefore inhibit their function. Indeed, specific chemical compounds, which efficiently block the activity or function of these viral proteins are now commercially available and in use worldwide (7–11).

Studies have demonstrated that administration of a mixture of 3 antiviral compounds (called combination chemotherapy or highly active retroviral therapy, HAART) can lead to significant reduction of viral load in vivo. Using 2 reverse transcriptase and 1 protease inhibitor in treatment naive patients, the serum HIV-1 RNA levels may be reduced to an undetectable level. How long this response will last in these patients remains an open issue. Several reports have described variant strains of HIV-1, among patients receiving combination chemotherapy (12–14).

In particular, patients who have been treated with 1 antiviral inhibitor alone in the past appear to already carry a virus

† Deceased.

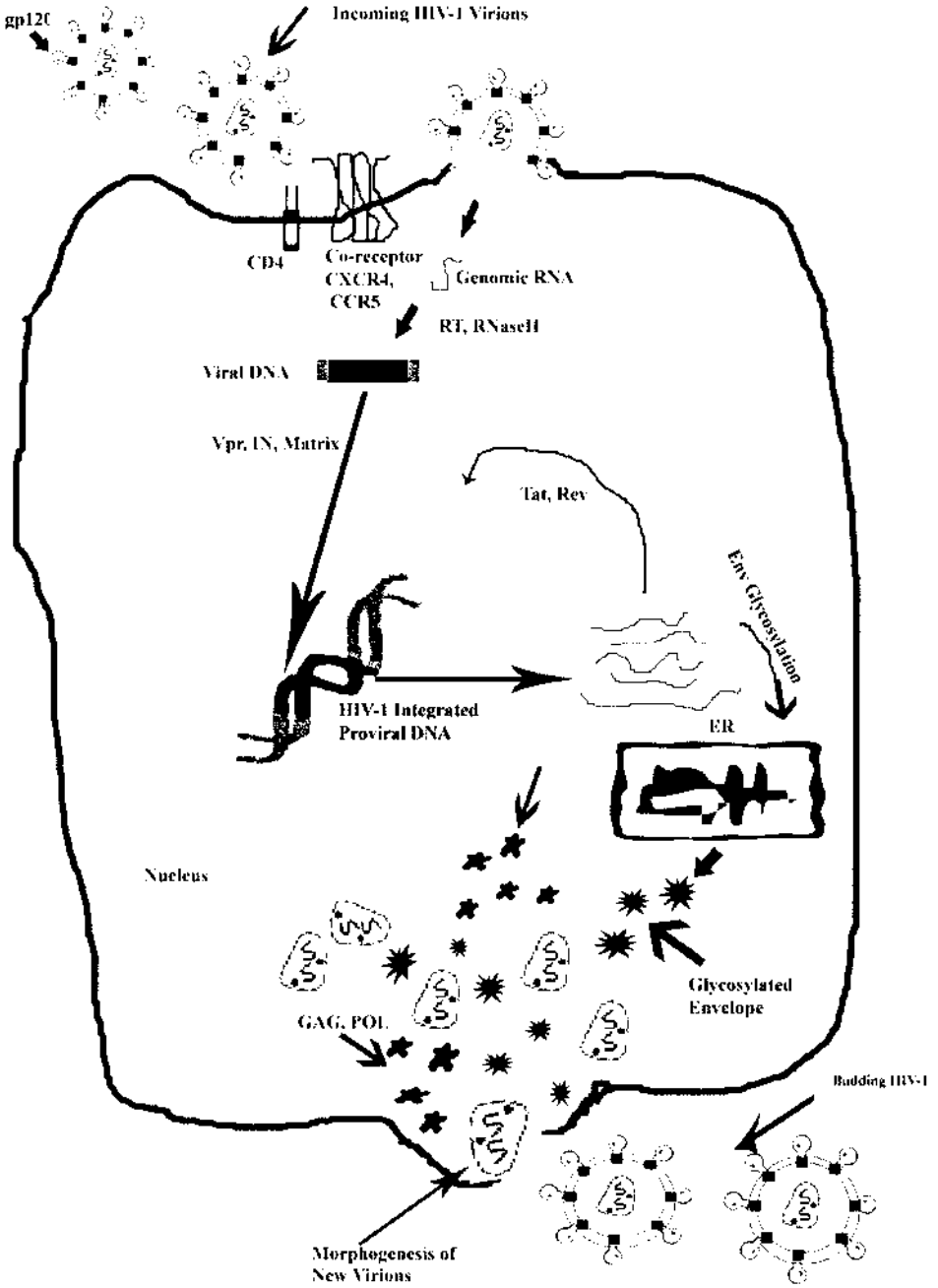

Figure 1 Life cycle of HIV-1. The life cycle of the human immunodeficiency virus type I is similar to that of all retroviruses studied. HIV-1 attaches to the target cell mainly by binding to the CD4 molecule and chemokine receptors. After fusion of the viral and cellular membranes, retroviral core particles are released into the cytoplasm. The RNA genome is converted into a double-stranded DNA by the viral reverse transcriptase (RT) and ribonuclease H (RNaseH) and actively transported into the nucleus, probably aided by the viral protein vpr. The viral DNA is integrated into the genome of the host cell by the viral integrase (IN). The integrated DNA form of the virus is called the provirus. In contrast to other retroviruses, transcription and RNA splicing of the provirus is regulated by viral accessory proteins. For example, the viral protein Tat must to bind to a specific sequence in the HIV genome (termed TAR) to enable highly efficient transcription of the provirus. Rev is required to control RNA splicing and the transport of RNAs into the cytoplasm. Finally, in the cytoplasm, virus core particles are assembled by encapsidating full-length genomic viral RNAs (recognized by specific encapsidation sequences). At the cell membrane, virus particle assembly is completed by the interaction of the core with the viral membrane proteins and new particles ''bud'' (are released) from the infected cell. For more details regarding regulatory proteins, see also Fig. 5. Env, envelope; ER, endoplasmatic reticulum.

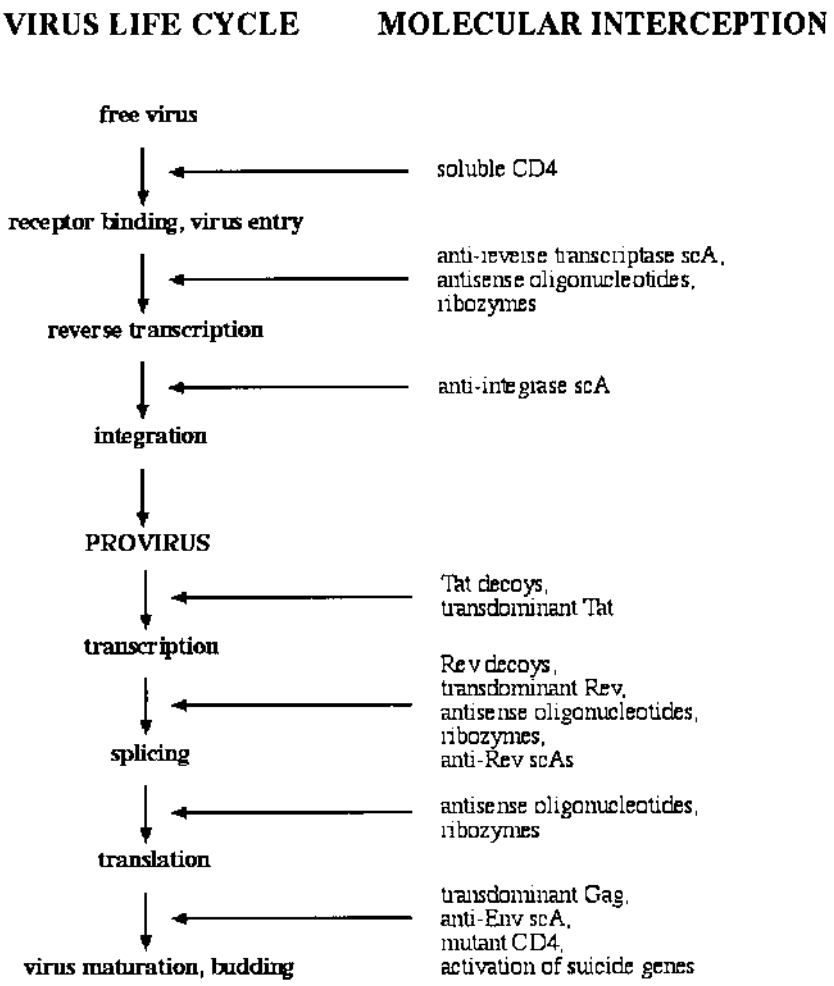

Figure 2 Overview of possible genetic targets to block HIV-1 replication. The different approaches are described in detail in the text.

strain, which is resistant to 1 of such compounds and, therefore, are partially resistant to combination therapy. Such virus strains have a higher chance to further mutate and escape the inhibitory effects of the other chemical compounds. In particular, the viral enzyme reverse transcriptase, which converts the viral RNA genome into a double-stranded DNA, does not have proofreading capabilities. On average, it inserts at least 1 incorrect nucleotide into the viral genome per replication cycle. This error rate is 1 million times higher than that of the cellular DNA polymerase I, which is the main enzyme for the replication of the eukaryotic genome. This high mutation rate explains why drug-resistant virus mutants emerge rapidly in HIV-1-infected patients (15). This high mutation rate also explains why new mutant viruses continuously arise, which are ''new'' and, therefore, not recognized and inactivated by the immune system. Consequently, in the clinically latent stage of HIV-1 infection, high virus loads persist, which consistently change their genetic outfit to escape drugs that inhibit virus replication and the immune system (16–18).

In summary, the clinical application of all drugs for the treatment of AIDS has not led to a cure of the disease and even the new combination therapy may only halt the development of AIDS in infected people temporarily, as new drug-resistant variants of the HIV-1 virus start to emerge. Thus, efforts are underway in many laboratories to develop alternative therapeutics.

III. GENETIC ''BULLETS'' TO BLOCK HIV-1 REPLICATION

The primary target cells for HIV-1 are cells of the hematopoietic system, in particular, $CD4^+$ T lymphocytes and macrophages. During HIV-1 infection, these cells are destroyed by the virus leading to immunodeficiency among infected individuals. To prevent the destruction of the cells of the immune system, many efforts are now underway to make such cells resistant to the HIV-1 virus. This approach has been termed

''intracellular immunization'' (19). In particular, the development of genetic agents, which attack the virus at several points simultaneously inside the cell and/or are independent from viral mutations, has gained great attention.

Such potential agents, also termed ''genetic antivirals'' should have 4 features, to overcome the shortcomings of conventional treatments. First, they should be directed against a highly conserved moiety in HIV-1, which is absolutely essential for virus replication eliminating the chance that new mutant variants arise, that can escape this attack. Second, they have to be highly effective, greatly reducing or, ideally, completely blocking the production of progeny virus. Third, they have to be nontoxic. A fourth criterion, which also should not be overlooked is that the antiviral agent has to be tolerated by the immune system. It would not make much sense to endow immune cells with an antiviral agent, that elicits an immune response against itself leading to the destruction of the HIV-1-resistant cell after a short period of time.

In the past few years, many strategies have been developed and proposed for clinical application to block HIV-1 replication inside the cell (see also Fig. 2). Such strategies use either antiviral RNAs or proteins. They include antisense oligonucleotides, ribozymes, RNA decoys, transdominant mutant proteins, products of toxic genes, and single-chain antigen-binding proteins (20–32). Antiviral strategies that employ RNAs have the advantage that they are less likely to be immunogenic than protein-based antiviral agents. However, protein-based systems have been engineered that use inducible promoters that only become active upon HIV-1 infection.

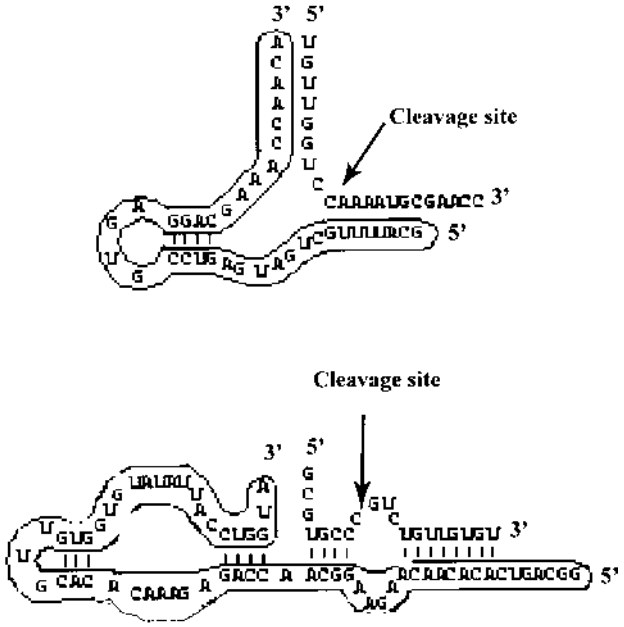

Figure 3 Schematic representation of 2 ribozymes to block HIV-1 replication. The structures shown are paired with actual HIV-I target sequences. (Top) A hammerhead ribozyme pairs specifically with a sequence in the gag region of the HIV-1 genome. (Below) A hairpin ribozyme designed to bind to and cleave the 5′ end of the viral genome, abolishing the reverse transcription and integration of progeny virus.

A. RNA-based Inhibitors of HIV-1 Replication

1. Antisense RNAs and Ribozymes

It is very well known that prokaryotes and bacteriophages express antisense RNAs, which provide regulatory control over gene expression by hybridizing to specific RNA sequences (33). In animal cells, artificial antisense oligonucleotides (RNAs or single-stranded DNAs) have been successfully used to selectively prevent expression of various genes (e.g., oncogenes, differentiation genes, viral genes, etc.) (33,34). Furthermore, the presence of double-stranded RNA inside the cell can induce the production of interferon and/or other cytokines stimulating an immune response. Indeed, it has also been reported that the expression of RNAs capable of forming a double-stranded RNA molecule with the HIV-1 RNA (antisense RNAs) can significantly reduce the expression of HIV-1 proteins, and consequently the efficiency of progeny virus production (33–38).

Ribozymes are very similar to antisense RNAs (e.g., they bind to specific RNA sequences), but they are also capable of cleaving their target at the binding site catalytically. Thus, they have the advantage that they may not need to be overexpressed to fulfil their function. Certain ribozymes (e.g., hairpin and hammerhead ribozymes, require only a GUC sequence) (Fig. 3). Thus, many sites in the HIV-I genome can be targeted. However, several questions still remain to be answered: for example, it is unclear, (a) whether efficient subcellular colocalization can be obtained, in particular, in vivo, (b) whether the target RNA will be efficiently recognized due to secondary and tertiary folding of the target RNA, or (c) whether RNA-binding proteins would prevent efficient binding. Thus, more experimentation will be necessary to address these problems (39–52). Efforts are also underway to develop multimeric/multivalent ribozymes for targeting all major clades of HIV-1, besides optimizing expression potential of these labile moieties (53–55).

2. RNA Decoys

In contrast to ribozymes and antisense RNAs, RNA decoys do not attack the viral RNAs directly. RNA decoys are mutant RNAs that resemble authentic viral RNAs and have crucial functions in the viral life cycle. They mimic such RNA structures and decoy viral and/or cellular factors required for the propagation of the virus (39,56–64). For example, HIV-1 replication largely depends on the 2 regulatory proteins Tat and Rev. These proteins bind to specific regions in the viral RNA, the transactivation response (TAR) loop and the Rev response element (RRE), respectively. Tat binding to TAR is crucial in the initiation of RNA transcription, Rev binding to RRE is essential in controlling splicing, RNA stability, and the trans-

port of the viral RNA from the nucleus to the cytoplasm. These 2 complex secondary RNA structures within the HIV-1 genome appear to be unique for the HIV-1 virus and no cellular homologous structures have been identified. Thus, such structures appear to be valuable targets for the attack with genetic antivirals.

The strategy here is to endow HIV-1 target cells with genes that overexpress short RNAs containing TAR or RRE sequences. The rationale for this is to have RNA molecules within the cells in abundance, which will capture Tat or Rev proteins, preventing the binding of such proteins to their actual targets. Based on Tat nuclear localization capabilities, recently, a chimeric small nucleolar RNA-TAR decoy that localizes to the nucleolus and nucleoli of human cells has also been described (65,66). As such it will be possible to halt early HIV-1 gene expression. Encouraging in vitro data from combination anti-HIV-1 gene therapy approaches using multiple anti-HIV-1 gene therapy moieties (i.e., antisense, decoy and trans-dominant negative mutants) supports its possible future clinical applications (67,68). Combination gene therapy is envisioned as an alternative for HIV-1 therapy due to its labile genome (69). Consequently, HIV-1 replication is markedly impaired. This strategy has the advantage over antisense RNAs and ribozymes in that mutant Tat or Rev, which will not bind to the RNA decoys will also not bind to their actual targets. Thus, the likelihood that mutant strains would arise that would bypass the RNA decoy trap is low. However, it still remains to be elucidated, whether cellular factors also bind to Tat or Rev decoys, and whether overexpression of decoy RNAs would lead to the sequestering of the resulting protein–RNA complexes in the cell.

3. Small Inhibitory RNA

RNA interference (RNAi) is a newly recognized phenomenon in the field of HIV-1 gene therapy with potential promises (70). The inhibitory mechanism involves posttranscriptional gene silencing. RNAi exploits double-stranded ribonucleic acid (dsRNA) oligonucleotides to inhibit expression of the gene with sequence similarity to the input oligonucleotide (Fig. 4). A 21–25 mer dsRNA oligonucleotide is used to interfere with the messenger RNAs (mRNAs) of a specific gene. One of the major prerequisites for achieving RNA interference is a perfect and unique match between the dsRNA oligo with mRNA that produces protein. The dsRNA binds to the target mRNA and makes it incomompetent for translation to protein. At molecular levels, a 2-step process involves editing of the dsRNA oligo by a protein complex called DICER, an RNAse-like enzyme, followed by another complex, called the RNA inhibitory silencing complex, that digests mRNA with similar sequences and hence silences that gene of interest.

There could be 3 major potential avenues for RNAi technology in HIV-1 therapeutic development. HIV-1 infection could be controlled by reducing host cellular targets such as CD4, CXCR4, and CCR5 or by silencing early HIV-1 gene products, Tat and Rev. HIV-1 integrase and gag could also be potential targets due to their crucial role in integration and morphogenesis, respectively. Several recent in vitro studies

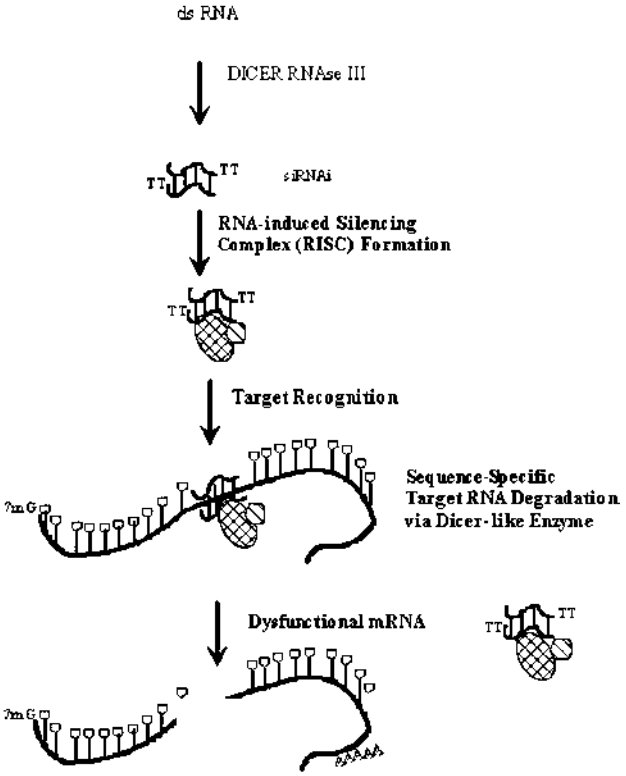

Figure 4 RNA interference model. The mechanism of action of dsRNA interference involves a key enzyme, RNAse III, commonly known as Dicer, that processes dsRNA to siRNA. Dicer generates siRNA duplexes having 5′-phosphate and free 3′-hydroxyl groups. This is followed by siRNA duplexes incorporation into siRNA-containing ribonucleoprotein complexes to generate RNA-induced silencing complexes (RISC) endonuclease. The RISC further mediates sequence-specific target RNA degradation.

targeting CD4, Tat, Rev, and Gag mRNA document the strength of this technology (71–76).

The in vitro findings of HIV-1 control by RNAi serve as a proof of principle that RNAi technology can be employed to suppress multiple steps in the HIV-1 life cycle (77). Moreover, targeting of HIV-1 coreceptors, such as CXCR4, CCR5, APJ, and a number of others, might be a promising future for this technology in HIV-1 gene therapy. An advantage of such interference would be its lowered effect on normal immune functions, one of the hurdles in conventional gene therapy.

RNAi technology will be facing several challenges before it acceptance in clinical scenarios. A major issue will be the stability of small oligos used for therapeutic purposes. In spite of such challenges, strength of this technology is the availability of data through the Human Genome Project to cross-reference redundancy of siRNAs selected for silencing purposes. Development of a vector-based strategy to target a combina-

tion of viral and cellular genes, essential for HIV-1 infection, will further add to the strength of this therapeutic intervention.

B. Protein-based Inhibitors of HIV-1 Replication

1. Transdominant Mutant Proteins

During HIV-1 replication, several regulatory proteins are essential for viral gene expression and gene regulation. Mutant forms of such proteins greatly reduce the efficiency of viral replication (78). Transdominant (TD) mutants are genetically modified viral proteins that still bind to their targets but are unable to perform their actual function. They compete with the corresponding native, wild-type protein inside the cell. The competition of several TD proteins with the wild-type counterpart has been shown to greatly reduce virus replication, especially when such TD mutants are expressed from strong promoters (the cytomegalovirus immediate early promoter, CMV-IE) (19,26,79–88).

For example, transcription from the HIV-1 long terminal repeat (LTR) promoter is dependent on the Tat protein. Mutant Tat proteins, which still bind to the nascent viral RNA, but which are unable to further trigger RNA elongation of transcription greatly reduce the production of HIV-I-RNAs and consequently the production of progeny virus. In a similar way, mutant Rev proteins interfere with regulated posttranscriptional events and also greatly reduce the efficiency of virus replication in an infected cell.

Although TD mutants have been shown to be effective in vitro, it still remains unclear how long cells endowed with such proteins will survive in vivo. An exciting area of transdominant protein research is exploring inhibitors of host proteins that interact with HIV-1 proteins. One such report using a protein that interacts with HIV-1 integrase [i.e., integrase interactor 1 also known as hSNF5] showed inhibitory effects on HIV-1 replication (89) There is a significant possibility that peptides of such proteins will be displayed via HLA leading to the destruction of the HIV-I-resistant cell by the patient's own immune system. There is, however, the potential to express such proteins from the HIV-1 LTR promoter, which only becomes activated upon HIV-1 infection. However, this would rule out that a TD Tat can be used, because TD Tat may also abolish its own expression. Even if other TD proteins are expressed from inducible promoters, it still remains unclear, whether such inducible promoters are really silent enough so that no protein is made (and no immune response) as long as there is no viral infection.

2. Toxic Genes

Another approach to reduce the production of progeny virus is to endow the target cells of HIV-1 with toxic genes, which become activated immediately after virus infection. The activation of the toxic gene leads to immediate cell death; therefore, no new progeny virus particles can be produced. Theoretically, this would lead to an overall reduction of the virus load in the patient. In vitro experiments have shown that the production of HIV-1 virus particles was indeed reduced, if target cells had been endowed with genes coding for the herpes simplex virus thymidine kinase or a mutant form of the bacterial diphtheria toxin protein. Such genes were inserted downstream of the HIV-1 LTR promoter, which only becomes activated upon HIV-1 infection, when the viral Tat protein is expressed (90–94).

Besides the question regarding the ''silence'' of the HIV-1 LTR promoter without Tat (discussed above), the main problem with this approach is the actual number of cells that carry a toxic gene present in the patient. Because the HIV-1 virus will not only infect cells that carry the toxic gene, but also many other cells of its host, this approach may only ''slow down'' virus replication for a short period of time until all cells that carry the toxic genes undergo self-destruction upon infection.

3. CD4 as Decoy

The CD4 molecule is the major receptor for the HIV-1 virus for entry into T lymphocytes. Thus, in a similar way to RNA decoys, mutant CD4, which stays inside the endoplasmatic reticulum has been shown to inactivate HIV-1 envelope maturation, preventing formation of infectious particles. In another approach, soluble CD4 has been used to block the envelope of free extracellular virus particles and to prevent binding to fresh target cells (95). However, the question remains if soluble and/or mutant CD4 in the blood of the patient will also serve as a trap for natural CD4 ligands leading to the impairment of important physiological functions (96–98).

4. Single-chain Antibodies

Single-chain antibodies (scA) have originally been developed for *Escherichia coli* expression to bypass the costly production of monoclonal antibodies in tissue culture or mice (99,100). They comprise only the variable domains of both the heavy and the light chain of an antibody. These domains are expressed from a single gene, in which the coding region for these domains are separated by a short spacer sequence coding for a peptide bridge, which connects the 2 variable domain peptides. The resulting scA [also termed single-chain variable fragment (scFv)] can bind to its antigen with similar affinity as a Fab fragment of the authentic antibody molecule.

scFvs have been developed by our group and others to combat HIV-1 replication, when expressed intracellularly (32,97,101–107). Both pre- (e.g., integrase, reverse transcriptase, matrix protein) and post- (e.g., Rev and Tat) integration sites of the viral life cycle have been targeted, with varying success (108). An HIV-1 coreceptor CXCR4 scFv has shown potent inhibitory effects on HIV-1 replication (109), and future development of CCR5 scFvs will further assist in HIV-1 control. Further studies using constructs combining multiple scFvs for potential synergistic antiviral potency are under development and should be of importance in developing robust anti-HIV-1 molecular therapeutics.

IV. GENETIC ''GUNS'' TO DELIVER GENETIC ANTIVIRALS

In all therapeutic approaches listed above, the therapeutic agent cannot be delivered directly to the cell. Instead, the

corresponding genes have to be transduced to express the therapeutic agent of interest within the target cell. Genes can be delivered using a large variety of molecular tools. Such tools range from nonviral delivery agents (liposomes or even naked DNA) to viral vectors. Because HIV-1 remains and replicates in the body of an infected person for many years, it will be essential to stably introduce therapeutic genes into the genome of target cells for either continuous expression or for availability upon demand. Thus, gene delivery tools such as naked DNA, liposomes, or adenoviruses (AV), which are highly effective for transient expression of therapeutic genes, may not be useful for gene therapy of HIV-1 infection.

Adeno-associated virus (AAV), a nonpathogenic single-stranded DNA virus of the parvovirus family, has recently gained a great deal of attention as a vector because it is not only capable of inserting its genome specifically at one site at chromosome 19 in human cells, but it is also capable of infecting nondividing cells. However, vectors derived from AAV are much less efficient and lose their ability to target chromosome 19 (110,111). Another shortcoming of AAV is the need for it to be propagated with replication-competent AV because AAV alone is replication defective. It also remains to be shown how efficiently AAV vectors transduce genes into human hematopoietic stem cells and/or mature T lymphocytes and macrophages. Because gene therapy of HIV-1 infection may also require multiple injections of the vector (in vivo gene therapy, see below) or of ex vivo manipulated cells, it also remains to be shown whether even small amounts of contaminating AV, which is used as a helper agent to grow AAV, will cause immune problems.

The most efficient tools for stable gene delivery are retroviral vectors (112–118), which stably integrate into the genome of the host cell, as this is a part of the retroviral life cycle (Fig. 1). This is why first virus-based gene delivery systems have been derived from this class of viruses, and continued development toward this class of safer vectors will lead to more suitable gene therapy system (119,120). This is also why they are being used in almost all current human gene therapy trials, including ongoing clinical AIDS trials.

Retroviral vectors are basically retroviral particles that contain a genome in which all viral protein-coding sequences have been replaced with the gene(s) of interest. As a result, such viruses cannot further replicate after 1 round of infection. Furthermore, infected cells do not express any retroviral proteins, which makes cells that carry a vector provirus (the integrated DNA form of a retrovirus) invisible to the immune system (112–118).

A. Retroviral Vectors Derived from C-Type Retroviruses

All current retroviral vectors used in clinical trials have been derived from murine leukemia virus (MLV), a C-type retroviruses with a rather simple genomic organization (Fig. 5). MLV contains only 2 gene units, which code for the inner core structure proteins and the envelope protein, respectively. It does not contain regulatory genes such as HIV-1. Thus, the construction of safe gene delivery systems is rather simple and straightforward. Such delivery systems consist of 2 components: the retroviral vector, which is a genetically modified viral genome, that contains the gene of interest replacing retroviral protein coding sequences, and a helper cell that supplies the retroviral proteins for the encapsidation of the vector genome into retroviral particles (Fig. 5). Modern helper cells contain separate plasmid constructs, which express all retroviral proteins necessary for replication. After transfection of the vector genome into such helper cells, the vector genome is encapsidated into virus particles (due to the presence of specific encapsidation sequences). Virus particles are released from the helper cell carrying a genome containing only the gene(s) of interest (Fig. 5). Thus, once established, retrovirus helper cells can produce gene transfer particles for very long time periods (e.g., several years). In the last decade, several retroviral vector systems have also been derived from other C-type chicken retroviruses (112,114,118).

B. Retroviral Vectors Derived from HIV-1

Retroviral vectors derived from MLV have been shown to be very useful to transfer genes into a large variety of human cells. However, they poorly infect human hematopoietic cells, because such cells lack the receptor, which is recognized by the MLV envelope protein. Furthermore, retroviral vectors derived from C-type retroviruses are unable to infect quiescent cells: such viruses (and their vectors) can only establish a provirus after 1 cell division, during which the nuclear membrane is temporarily dissolved. Thus, efforts are underway in many laboratories to develop retroviral vectors from lentiviruses [e.g., HIV-1 or the simian immunodeficiency virus (SIV), which are able to establish a provirus in nondividing cells (although the mechanism by which these viruses penetrate the nucleus is not fully understood)] (121). However, the fact that lentiviruses contain several regulatory proteins, which are essential for virus replication, makes the construction of lentiviral packaging cells more complicated. Furthermore, the fact that the lentiviral envelope proteins (e.g., that of HIV-1) can cause syncytia and/or that some viral regulatory proteins are toxic to the cells further hampers the development of stable packaging lines.

The ''envelope problem'' has been solved by generating packaging cells, which express the envelope protein of MLV or the envelope of vesicular stomatitis virus (VSV). Such envelope proteins are efficiently incorporated into lentiviral particles. The second and major problem for generating stable packaging lines is the toxicity of some retroviral regulatory proteins to the cell. Thus, retroviral vectors can only be generated in transient systems: 293T cells (human embryonic kidney cell line, highly susceptible for transfecting DNAs) are simultaneously transfected with all plasmids constructs to express the particle proteins and the vector genome. Figure 6 shows plasmid constructs used to make HIV-1-derived packaging cells. Vector virus can be harvested from the transfected cells for a limited time period and can be used to infect fresh target cells. Although this gene transfer system has been

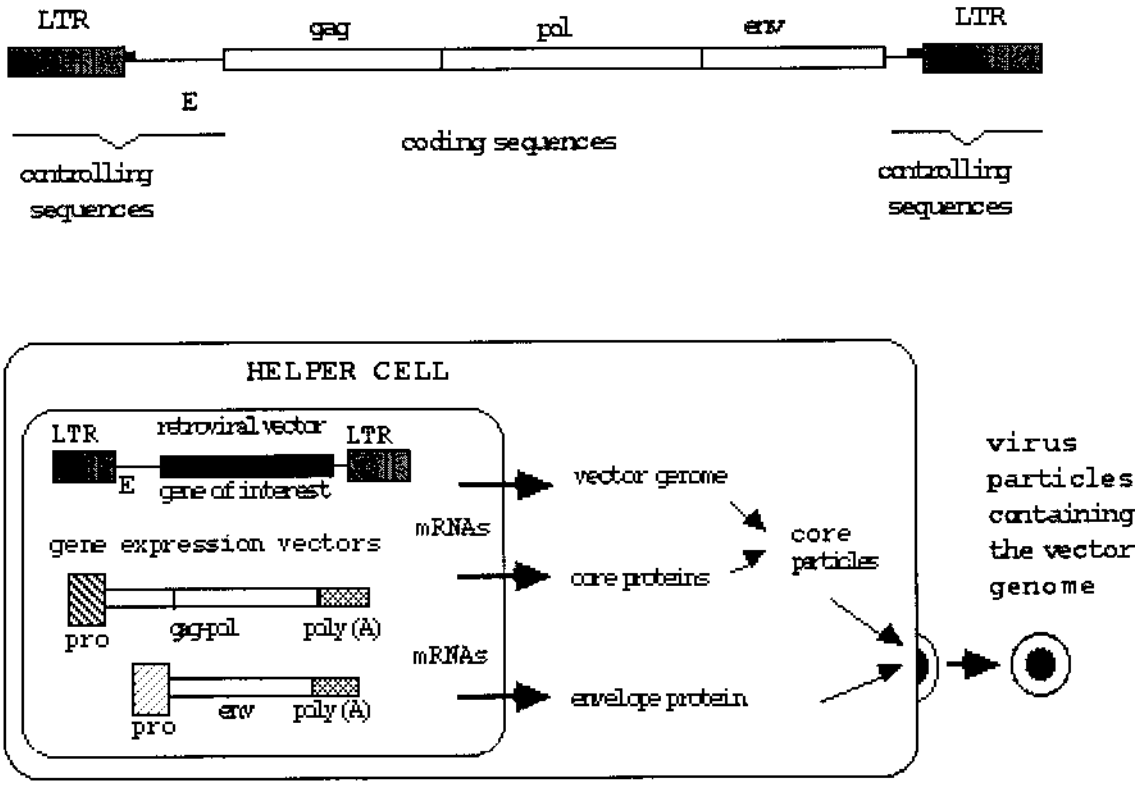

Figure 5 Retroviral helper cells derived from C-type retroviruses. A C-type retroviral provirus (the DNA intermediate of a retrovirus is shown on the top). The protein coding genes (gag-pol and env) are flanked by cis-acting or controlling sequences, which play essential roles during replication. (Below) In a retroviral helper cell, the retroviral protein coding genes, which code for all virion proteins, are expressed (ideally) from heterologous promoters (pro) and polyadenylated via a heterologous polyadenylation signal sequence [poly(A)]. To minimize reconstitution of a full-length provirus by recombination, the gag-pol and env genes are split to different gene expression vectors. In the retroviral vector, the viral protein coding sequences are completely replaced by the gene(s) of interest. Because the vector contains specific encapsidation sequences (E), the vector genome is encapsidated into retroviral vector particles, which bud from the helper cell. The virion contains all proteins necessary to reverse transcribe and integrate the vector genome into that of a newly infected target cell. However, because there are no retroviral protein coding sequences in the target cell, vector replication is limited to 1 round of infection. LTR, long terminal repeat.

shown to be functional, it is not highly efficient and better packaging cells still need to be developed. In addition, many questions regarding the safety of such vectors still need to be addressed. It is known that plasmid DNAs can recombine with each other very efficiently immediately after transfection. Thus, the question needs to be addressed, whether there is a chance that replication-competent viruses arise by recombination, which may cause a disease in gene-transduced patients.

C. Cell Type-specific Retroviral Vectors

All retroviral vectors currently used in human gene therapy trials contain the envelope protein of amphotropic (ampho) MLV or VSV. Ampho-MLV and VSV have a broad host range and can infect various tissues of many species, including humans. Thus, the use of vectors containing such envelope proteins enables the transduction into many different human tissues. However, due to this broad host range, gene transfer has to be performed ex vivo. If injected directly into the bloodstream, the chances that the vector particles would infect their actual target cells are very low. Furthermore, such vector particles may infect germ line cells (which are continuously dividing). Thus, the target cells have to be isolated and the gene transfer is being performed in tissue culture. Gene transduced cells are then selected and reintroduced into the patient.

However, this protocol has major shortcomings in regard (not only) to gene therapy of HIV-1 infection. First, it is very expensive and requires highly trained personal. Second, human cells, which are kept in tissue culture change their physiological behavior and/or take up fetal bovine proteins (a component of the tissue culture medium) and display bovine peptides via histocompatibility antigen (HLA) on the cell sur-

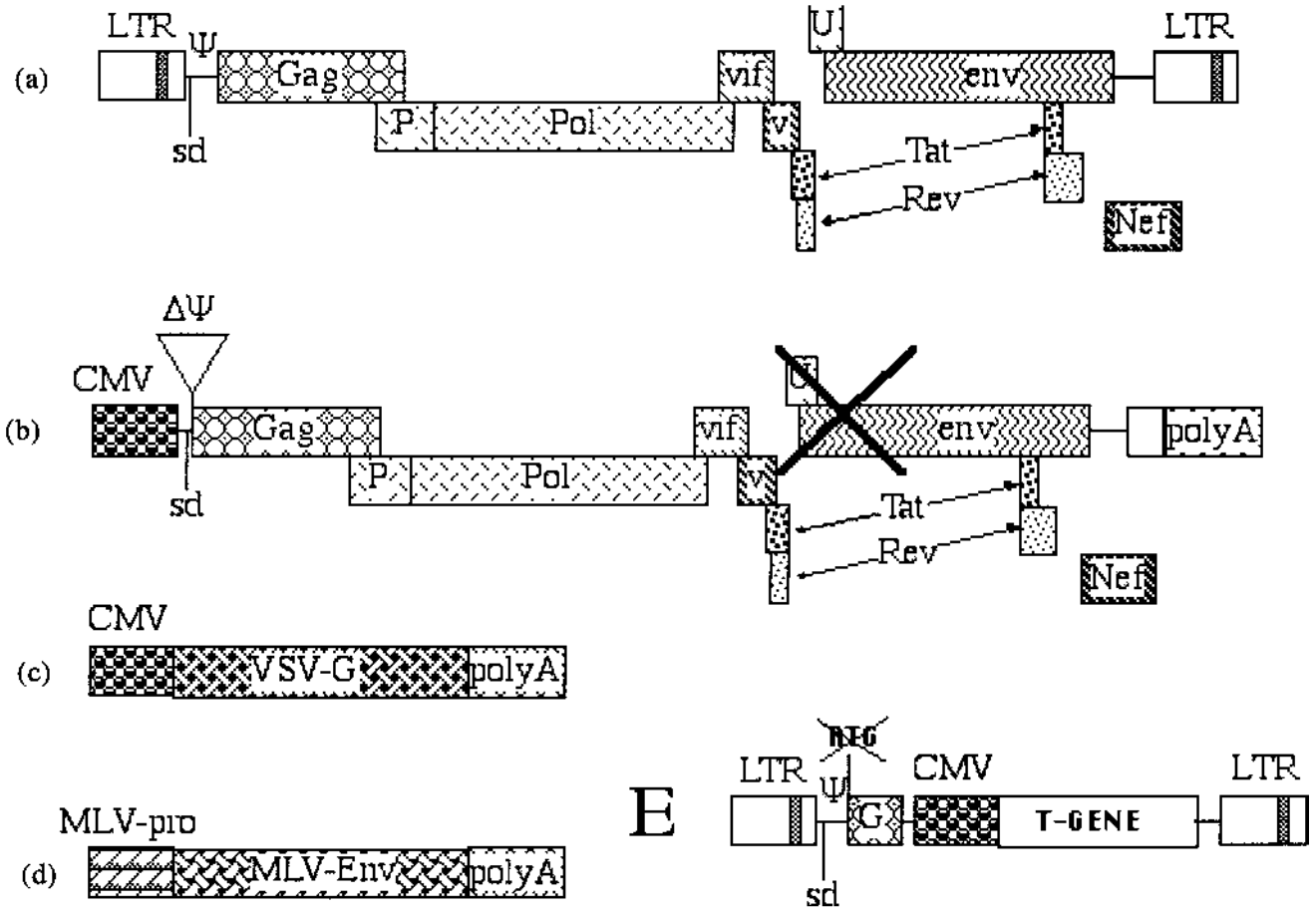

Figure 6 Retroviral packaging system derived from HIV-1. (a) A provirus of HIV-1 is shown on the top. (b–d) These show plasmid constructs to express pseudotyped HIV-1 retroviral particles, and (E) shows a plasmid construct to encapsidate and transduce genes with an HIV-1 vector (the plasmid sequences to propagate such constructs in bacteria are not shown). Besides the genes encoding for HIV-1 proteins, which form the core of the virus (e.g., Gag, structural core proteins; P, protease; Pol, reverse transcriptase and integrase) and the envelope (e.g., env, envelope protein), the HIV-1 genome also codes for several regulatory proteins, which are expressed from spliced mRNAs and have important functions in the viral life cycle. (b) plasmid construct to express the core and regulatory proteins. To avoid encapsidation and transduction of genes coding for such proteins, the following modifications have been made: the 5′ LTR promoter of the HIV-1 provirus has been replaced with the promoter of cytomegalovirus (CMV) to enable constitutive gene expression, the 3′ LTR has been partially replaced with the polyadenylation signal sequence of simian virus 40 (polyA), the encapsidation signal has been deleted ($\Delta\psi$), and the reading frame for the envelope and vpu genes have been blocked. (c) and (d) show plasmid constructs to express the envelope proteins of the vesicular stomatitis virus (VSV-G) or the envelope protein of murine leukemia virus (MLV), respectively. In the absence of HIV-1 envelope proteins, which are rather toxic to the cell, HIV-1 efficiently incorporates the envelope proteins of VSV or MLV into virions. The use of such envelopes also further reduces the risk of the reconstitution of a replication-competent HIV-1 by homologous recombination between the plasmid constructs. (E) shows a retroviral vector used to package and transduce a gene of interest (T gene) with HIV-1-derived vectors. Because the encapsidation sequence extends into the Gag region, part of the gag-gene (G) has been conserved in the vector. However, the ATG start codon has been mutated. The gene of interest is expressed from an internal promoter, because the HIV-1 LTR promoter is silent without Tat. sd, splice donor site.

face. Consequently, such cells become immunogenic and are eliminated by the immune system of the patient. To bypass such ex vivo protocols, efforts are now underway in many laboratories to develop cell type-specific gene delivery systems, which would involve injecting the gene delivery vehicle directly into the patient's bloodstream or tissue of interest. In the past few years, several attempts have been made to develop cell type-specific gene delivery tools, again with retrovirus-derived vectors leading the field.

The cell type specificity of a virus particle is determined by the nature of the retroviral envelope protein, which mediates the binding of the virus to a receptor of the target cell (122). Thus, experiments have been initiated in several laboratories to modify the envelope protein of retroviruses in order to alter the host range of the vector. One of the first attempts to specifically deliver genes into distinct target cells has been performed in the laboratory of Dr. H. Varmus. Using retroviral vectors derived from avian leukosis virus, these investigators incorporated the human CD4 molecule into virions to specifically transduce genes into HIV-1-infected cells (123). However, such particles were not infectious for unknown reasons. Recent reports indicate that MLV particles that carry CD4 can infect HIV-1-infected cells, although at very low efficiencies (124).

In another attempt to target retroviral particles to specific cells, Roux et al. have shown that human cells could be infected with eco-MLV, if they added 2 different antibodies to the virus particle solution (125,126). The antibodies were connected at their carboxy termini by streptavidine. One antibody was directed against a cell surface protein, the other antibody was directed against the retroviral envelope protein (125,126). Although this approach was not practical (infectivity was very inefficient and was performed at 4°C), these experiments showed that cells that do not have an appropriate receptor for a particular virus can be infected with that virus, if binding to the cell surface had been facilitated. These data also indicated that antibody-mediated cell targeting with retroviral vectors was possible.

To overcome the technical problems of creating an antibody bridge, it was logical to incorporate the antibody directly into the virus particle. However, complete antibodies are very bulky and are not suitable for this approach. The problem has been solved using single-chain antibody technology (127–130). Using hapten model systems, it has been shown that retroviral vectors that contained scAs fused to the envelope are competent for infection (128,129). Retroviral vector particles derived from spleen necrosis virus (SNV, an avian retrovirus) that display various scAs against human cell surface proteins are competent for infection on human cells that express the antigen recognized by the antibody (127,128,130).

Most recently, it became possible to use scA-displaying SNV to introduce genes into human T cells with the same high efficiency obtained with vectors containing wild-type envelope. In all such experiments, the wild-type envelope of SNV had to be copresent in the virus particle to enable efficient infection of human cells. However, because SNV vector particles with wild-type SNV envelope do not infect human cells at all, this requirement is not a drawback for using such vector particles for human gene therapy (131,132).

Although successful gene transfer using scA-displaying MLV vector particles has been reported from 1 laboratory (133), further experimentation in the laboratories of several other investigators revealed that MLV-derived vector particles that display various scAs are not competent for infection in human cells (125,126,134–137). The difference between MLV and SNV cell-targeting vectors is certainly based on the different features of the wild-type envelope and the mode of virus entry (138). Moreover, wild-type ampho-MLV infects human hematopoietic cells extremely poorly, most likely due to the absence of an ampho-MLV receptor on such cells (139). Thus, MLV-derived vectors are certainly not the best candidates for human gene therapy of AIDS and alternative vectors need to be developed.

D. Other New Potential Vector Systems

Most recently, other interesting attempts have been made to combat HIV-1-infected cells. Recombinant VSV has been engineered, which lacks its own glycoprotein gene. Instead, genes coding for the HIV-1 receptor CD4 and a chemokine coreceptor, CXCR4, have been inserted. The corresponding virus was able to efficiently infect HIV-1-infected cells, which display the HIV-1 glycoprotein on the cell surface. Because VSV is a virus that normally kills infected cells, the engineered virus only infects and kills HIV-1-infected cells. It has been reported that this virus indeed reduced HIV-1 replication in tissue culture cells up to 10,000-fold (140). This novel approach to combat one virus with another will certainly gain a great deal of further attention. However, it remains to be shown how effective this approach will be in vivo. Will the ''antivirus'' succeed in eliminating a large load of HIV-infected cells before it will be cleared by the immune system? On the other hand, because HIV-1 preferentially kills activated immune cells, will it destroy the immune cells that are attempting to clear the body from its own ''enemy''? How will the body tolerate a virus that does not look like one because it carries human cell surface proteins on the viral surface? The answer to these and other questions are eagerly awaited (141).

V. POTENTIAL PROBLEMS

Even if we find a gene transfer system that can transduce enough cells within the body to inhibit virus replication significantly, there are many other questions that still remain to be answered. For example, it is not clear whether a cell, which has been endowed with an HIV-1 resistance gene, will be able to fulfill its normal biological function in vivo. It has to be considered that to become resistant against HIV-1, the cells usually have to overproduce the corresponding HIV-1-resistance gene. Does this overproduction result in a loss of other functions (e.g., because the cell has to supply a significant part of its energy supply to the production of the HIV-1-resistance gene)? Will the body be able to eliminate all HIV-1-infected

cells or will the infected person become a lifelong carrier of the virus, which is still replicating in his or her body although at levels that cause no clinical symptoms due to the presence of HIV-1-resistance genes? Will the patient be capable of infecting new individuals? Finally, as genetic therapies can all be overcome with in vitro challenges of very high multiplicities of infection (MOIs) of HIV-I, will there be a difference in antiviral effects in peripheral blood vs. lymphoid tissues?

VI. ANIMAL MODELS

One of the major problems with any therapeutic agent against HIV-1 infection is the lack of an appropriate and inexpensive animal model system to test the efficiency of an antiviral agent. Because HIV-1 only causes AIDS in humans, it is difficult to test and evaluate the therapeutic effect of novel antiviral agents in vivo. Furthermore, the evaluation of the efficacy of a new drug is further complicated by the long latency period of the virus until the onset of AIDS (which can be 10 years or more). Although a virus similar to HIV-1 has been found in monkeys, the SIV results obtained with this virus do not necessarily reflect the onset of AIDS in humans caused by HIV-1. Furthermore, many antivirals that block HIV-1 are ineffective in blocking SIV. Thus, other animal model systems need to be developed to study the effect of anti-HIV-1 therapies.

Since the early 1990s, many strains of laboratory mice have been bred, which lack components of the immune system. Severe combined immunodeficient (SCID) mice are deficient in functional B and T lymphocytes. Thus, they are unable to reject allogeneic organ grafts (142–147). SCID mice have been used extensively to study human leukemia and other malignancies and for modeling human retroviral pathogenesis, including antiviral gene therapy. Furthermore, in the past few years, much progress has been made to transplant hematopoietic stem cells into SCID mice to mimic and study human hematopoiesis. It has been shown that transplantation of human hematopoietic cells into such mice can lead to the repopulation of the mouse's blood with human CD4- and CD8-positive cells. Thus, SCID mice appear also to be good candidates to develop mouse model systems for HIV-1 infection.

At present, 2 different SCID mice model systems are used to study the effect of anti-HIV-1 antiviral agents (148,149). The 2 systems are somewhat different because they represent different components of the human immune system. One is called the hu-PBL SCID model, the other is termed SCID-hu mouse.

In the hu-PBL SCID mouse system, human peripheral blood leukocytes are injected into the peritoneum of the animal. Thus, cells residing in such animals are mature $CD4^+$ and $CD8^+$ lymphocytes. The presence of activated and/or memory T cells ($CD45RO^+$ cells) has also been demonstrated. Such human cells can be recovered from various organs in the mouse (e.g., the spleen or lymph nodes). Because these animals contain human $CD4^+$ T cells, they can be infected easily with HIV-1. Even more, HIV-1 virus replicates in the animal leading to the depletion of $CD4^+$ T cells over a period of several weeks after infection. Thus, this experimental system is very valuable to test the effect of antiviral agents. For example, experiments have been performed to test the effect of monoclonal antibodies against the HIV-1 envelope protein (neutralizing the receptor-binding V3 domain). It has been shown that this antibody indeed could block the replication of the viral strain, for which the antibody was specific. This model system has also been used to test the efficiency of a vaccinia virus-derived vaccine.

In the SCID-hu mouse model system, various human fetal hematopoietic tissues (e.g., liver, lymph nodes, and/or thymus) are transplanted into the mouse (e.g., human fetal thymus and liver tissues are engrafted under the murine kidney capsule). It has been shown that normal thymopoiesis takes place for up to 1 year after implantation and human $CD4^+$ and $CD8^+$ T cells are found in the mouse blood at low levels. In contrast to the hu-PBL-SCID mouse, human cells are also found, that express the CD45RA antigen, which is considered a marker for ''naive'' T lymphocytes. Such mice can also be infected with HIV-1, although the injection of a high virus dose directly into the implant is necessary to establish an infection. However, once an infection has been established, the pathologic effects observed are similar to those observed in the thymuses of infected human adults, children, and fetuses. Moreover, a depletion of $CD4^+$/CD8-T cells is observed.

Both SCID mice model systems are very useful to study pathogenesis and the effect of anti-HIV-1 drugs and to test the effect of anti-HIV antiviral genes. However, one has to keep in mind that such model systems only represent a portion of the human immune system and HIV-1 also infects other cells in humans, such as dendrite cells, microvascular endothelial cells, and neurons in the brain. Thus, the pathogenesis observed in SCID mice certainly does not accurately reflect the pathogenesis in man. Moreover, it is not clear, whether SCID mice transplanted with human immune cells do have a functional immune system.

Recently, a group at the University of Maryland Biotechnology Institute has introduced an HIV-1 rat model (150,151). This particular transgenic animal model contains the entire genome of HIV-1, except for 2 genes involved in infectivity of the virus. These transgenic rats develop AIDS-like symptoms (i.e., weight loss, wasting syndrome, neurological complications) between 5 to 9 months. It is anticipated that HIV-1 rats will be more efficient than mouse models for molecular therapeutic studies.

VII. CLINICAL TRIALS

Recently, certain initial in vivo studies have been conducted for intracellular immunization against primate lentiviruses. A trans-dominant negative Rev protein (RevM10) has been studied in humans infected with HIV-1 by Dr. G. Nabel's group. In these initial studies, it was demonstrated that cells transduced with RevM10 had a significant longer half-life, as com-

pared with control cells, when reinfused into patients in different stages of disease. These early initial phase I trials were performed using MLVs, as well as microparticulate bombardment using a ''gene gun'' (152).

In addition, an very exciting study has been reported by R. Morgan's group in which an antisense construct to Tat and Rev genes in SIV was used to transduced T cells from rhesus macaques (153). The monkeys were then challenged with SIV intravenously. Of note, the animals with the transduced cells had significantly lower viral loads and higher CD4 counts, compared with control monkeys. Thus, this suggests, for the first time, that gene therapy against lentiviruses may have significant efficacy in vivo. Clearly, these are both preliminary studies in humans and in primates, which require more detailed evaluation. Other trials using a variety of different approaches are ongoing in initial phase I studies. According to data presented at The American Society of Gene Therapy 2002 meeting, HIV-1-infected subjects undergoing ex vivo $CD34^+$ stem cells transduction and transplantation continue to express anti-HIV-1 antisense RNA over 2 years postinfusion. This proprietary gene therapy product of Enzo Pharmaceutical is still under future development (154).

In general, gene therapy faced a major setback in 1999 due to the death of the first known gene therapy patient after receiving an experimental gene therapeutic for inherited liver disease. In the aftermath of this death, the National Institutes of Health and the Food and Drug Administration have instituted stricter guidelines for conducting and monitoring clinical trials with gene therapy (155,156).

In summary, considering all facts and problems of current gene transfer technologies, and considering our lack of knowledge regarding many functions of the immune system, how should we move forward with more phase I gene therapy trials to combat HIV-1? In spite of the lack of knowledge of many aspects of this disease, we have to remain optimistic and can only hope that one approach or the other will lead to measurable success toward the cure, or will at least significantly prolong the life expectancy of the infected person. Clearly, in addition to further exploring novel molecular therapeutics in vivo, significant attention must be placed toward answering critical basic science questions pertaining to ''intracellular immunization.''

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32

Cutaneous Gene Therapy for Skin and Systemic Disorders

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I. INTRODUCTION

The skin serves multiple functions owing to its unique and complex structure. The skin presents remarkable advantages as a tissue for developing innovative genetic therapeutic strategies. The skin is a readily accessible organ, which facilitates gene delivery, subsequent monitoring of transgene expression, and excision of small or large areas if required (1). Epidermal keratinocytes and dermal fibroblasts can be readily expanded in culture. In addition, keratinocytes have high proliferative potential and inherent biological characteristics that allow them to synthesize mature proteins from a vast array of transgenes (2).

There have been recent advances in the molecular characterization of many skin disorders, vector design, immune modulation, regulation of gene expression, administration, and in other aspects in the field of skin gene therapy. This rapid progress has made the skin a formidable target to develop and test a variety of gene therapy approaches to both cutaneous and systemic diseases.

II. SKIN: STRUCTURE AND FUNCTION

The skin, or integument, is the most superficial and largest organ of the body. Its functions are essential for the homeostatic balance of the organism. The skin provides protection against ultraviolet (UV) light, mechanic, thermic, and chemical insults, and also prevents excessive dehydration due to its relative water impermeability (3,4). It also acts as a physical barrier against microorganisms and is involved in the coordination of multiple immune responses (5,6). The skin is the major organ involved in sensory perception. For instance, it possesses tactile, pressure, pain and temperature receptors (7,8). In humans and in many other mammals the skin is essential in thermoregulatory responses (6,9,10). For example, heat conservation is aided by the presence of hair and adipose tissue in the hypodermis, and heat loss is increased by the evaporation of water through the skin's surface and by increasing the blood flux through the rich capillary plexus of the dermis (9). The skin also plays a definite role in the body's metabolism as an important store of energy in the form of triglycerides and in the synthesis of vitamin D (6,9).

The skin presents regional variations with regards to thickness, coloration, and presence of adnexa. For instance, the skin is thicker, presents lower levels of coloration, and has no hair follicles in the palms and soles. However, the basic structure of the skin is maintained in all body areas. The external part of the skin, the epidermis, is formed by a stratified squamous epithelium, which is composed in its majority by keratinocytes. Other cell types such as melanocytes, Langerhans cells, and Merkel cells are also found in this layer. The epidermis is divided into 2 major compartments: the basal (proliferative) and suprabasal (differentiation) compartments. The suprabasal compartment is further divided into different layers based on microscopic characteristics: the spinous layer, the granular layer, and the cornified layer (Fig. 1). The basal keratinocytes are attached to the basement membrane (BM) through specialized multiprotein junctional complexes called hemidesmosomes (11). The epidermis, a tissue in constant turnover, is sustained by permanent mitotic divisions in the basal compartment where stem cells reside (12). Recent studies suggest that the skin contains 3 distinct stem cell reservoirs: the interfollicular epidermis and the anagen hair follicle ger-

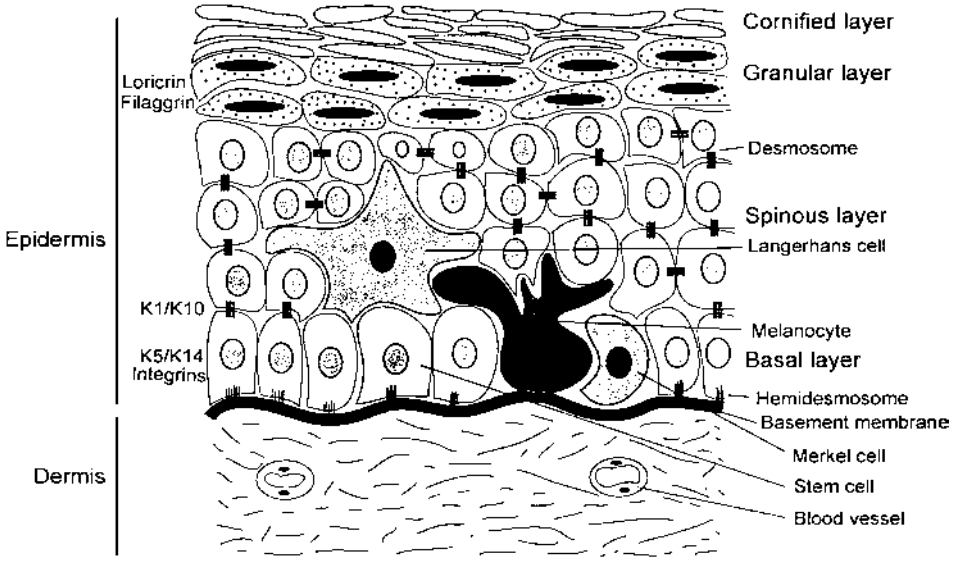

Figure 1 Schematic representation of the skin.

minal matrix where stem cells with a limited differentiation potential exist, and the upper outer root sheath (bulge), which contains potent reserve stem cells that act in the maintenance of not only the epidermis, but hair follicles and sebaceous glands (13). The cells in the basal layer undergo as series of maturational changes, and at some point, a given number move upward to the postmitotic suprabasal compartment where they undergo terminal differentiation. The basal layer is composed of a heterogeneous population of cells that can be classified according to their capacity for sustained growth into 3 subpopulations: (1). the holoclones or stem cells, which have the greatest reproductive capacity; (2). the paraclones or differentiated cells with a short replicative lifespan and limited growth capacity; and (3). the intermediate meroclones, which are the transitional stage between the holoclones and the paraclones (14). The expression of keratins, integrins, and involucrin is regulated during keratinocyte differentiation. Keratin 5 (K5), keratin 14 (K14), and integrins are expressed by basal cells, their expression is down-regulated as the cells detach from the BM and move upward to the suprabasal compartment where keratins 1 (K1) and 10 (K10) become highly expressed (15,16). Subsequently, as keratinocytes migrate toward the epidermal surface the expression of other markers, such as filagrin and loricrin, is detected (17,18). Most of the cellular organelles are degraded in the stratum granulosum, and the dead keratinocytes form the stratum corneum.

The dermis, a thick layer of fibroelastic dense connective tissue that supports and nourishes the epidermis, is composed of numerous blood and lymphatic vessels, sensory elements, and different cell types such as fibroblasts, macrophages, and lymphocytes. The hypodermis (subcutaneous layer or subcutis) is localized under the dermis, and contains adipose tissue and blood vessels. The adnexal structures such as sweat glands, sebaceous glands, and hair follicles are structures of ectodermal origin that form by invagination of the epidermic epithelium into the dermis, and sometimes the hypodermis (Fig. 1).

III. GENE DELIVERY TO THE SKIN: MAIN STRATEGIES AND GENE TRANSFER SYSTEMS

A. Main Strategies

The accessibility, visualization, and monitoring that made the skin an appealing target for genetic therapy has also allowed the development and use of strategies for gene transfer not possible in other tissues. The 2 main strategies currently used for gene delivery in skin disease models are the ex vivo and in vivo approaches.

1. The Ex Vivo Approach

In the ex vivo approach, the skin cells from the host are isolated and harvested after removal by biopsy. The cells are then grown in vitro where therapeutic gene transfer is performed. Finally, the altered cells are grafted back into the host (Fig. 2). This method offers some advantages because primary keratinocytes, including human, are receptive to gene modification, readily expanded in tissue culture under selective conditions, and easily grafted back into host (19–22, Buitrago and Roop, unpublished data). However, the ex vivo approach is disadvantageous because of its labor intensity and potential scarring.

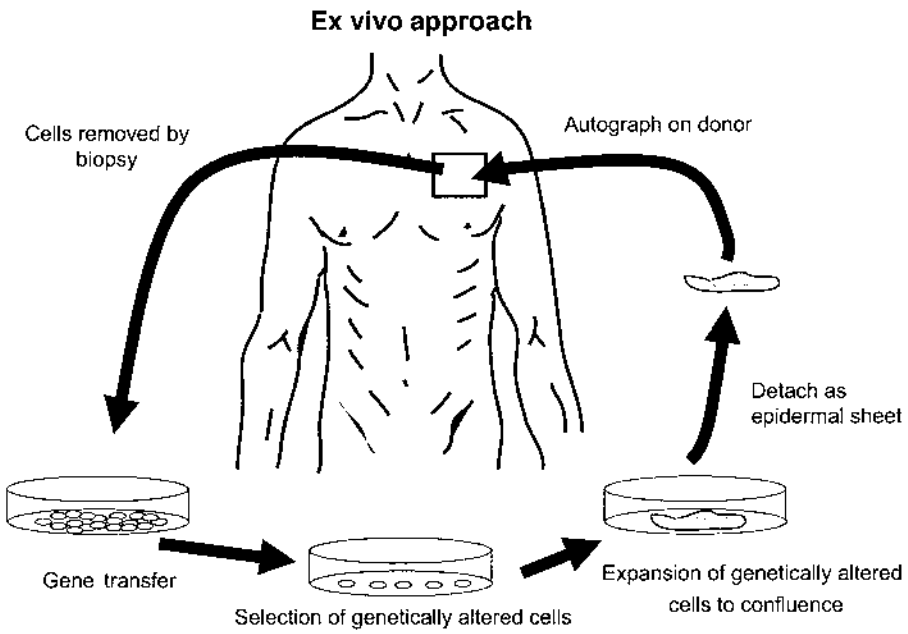

Figure 2 Ex vivo approach for cutaneous gene transfer.

2. The In Vivo Approach

In this approach, gene transfer is achieved through direct administration of genes by different modalities, such as naked DNA or in vectors of nonviral or viral origins (Fig. 3). The in vivo approach is favored over the ex vivo approach as the need for cell culture and surgery are bypassed, making it technically, clinically, and economically advantageous (19). In spite of these advantages, the direct in vivo approach is still limited by the low levels of transduction frequency for stem cells that leads to transient expression of the transferred gene.

B. Gene Transfer Systems in Cutaneous Gene Therapy

The first step in effective gene therapy is the ability to deliver the corrective gene/genetic material to the correct tissue with high efficiency. The skin can be specifically targeted by transduction in culture, intradermal injection, topical application, or other methods. A variety of specific techniques and vectors for skin gene delivery have been developed, and they can be classified in 2 large categories: viral and nonviral.

1. Viral Gene Transfer Vectors

Currently, viruses provide the most efficient means of delivering genes to target cells. Several classes of viruses have been successfully used for gene delivery and expression in the skin (Table 1). Viral vectors are efficient for gene transfer in cell culture as part of the ex vivo approach. In addition, they have also yielded positive outcomes when administered topically or by direct injection during the in vivo approach (23,24). As reviewed by Ghazizadeh and Taichman (25), there are 6 main factors to consider in a viral system for efficient gene transfer: ability for high titer generation, cargo-carrying capacity, capacity to transduce dividing and/or nondividing cells, integration properties, vector antigenicity, the ease with which clinical grade vectors can be prepared free of replication-competent virus, and the length of time of gene expression required (25). Some disadvantages should also be considered when working with viral vectors. Because the expression of the delivered genes depends heavily on the integration site, it is important to understand that all the integrative viral vectors have the limitation of their lack of true site-specific integration. Furthermore, down-regulation of the introduced genes by epigenetic mechanisms represents another significant obstacle. To overcome these obstacles, several strategies have been developed including use of insulator elements, and the bacterial tetracycline regulatory system (26–28).

a. Adenoviruses. These vectors have been widely used for skin gene transfer. Most of these vectors have been rendered replication deficient by deletion of their E1A and E1B essential genes (29,30). The development of ''gutless'' adenoviral vectors in which more viral genes are stripped has increased their cargo capacity and minimized the toxicity of viral products to target cells (31). Adenovirus vectors can carry up to 35 kb of foreign DNA, and the viral particles can be produced at high titers. They can infect a wide variety of cell types, and both replicating and nonreplicating cells. In addition, safety precedents already exist because adenovirus-based vaccines have been used in patients without any major side effect (32).

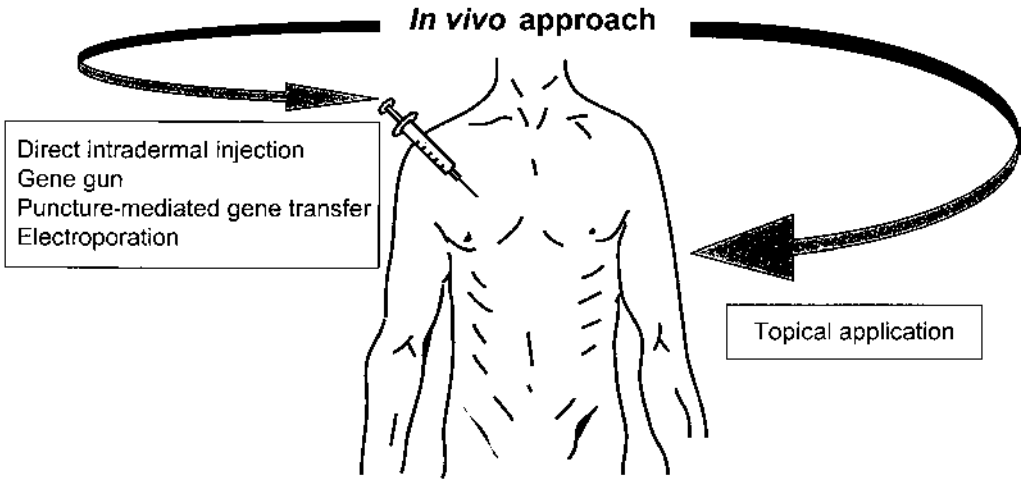

Figure 3 In vivo approach for cutaneous gene transfer.

Adenoviral transgene expression has been detected in all cell types of both dermis and epidermis when injected subcutaneously (19). However, adenoviruses are highly antigenic, and because they do not integrate into the host genome, only transient expression of the delivered gene is achieved most of the time. Therefore, adenoviral vectors are not ideal for sustained expression in a regenerating tissue such as the epidermis, making their use to correct inherited skin diseases limited. Nonetheless, it has been recently reported that recombinant adenovirus vectors carrying the xeroderma pigmentosum A (*XPA*) and C (*XPC*) genes achieved long-term expression, as well as long-term restoration of biological activity (up to 2 months) in XP-A and XP-C immortalized and primary fibroblast cell lines (33). Adenoviral vectors have been successfully used in applications such as DNA vaccination (34), anticancer therapy (35), and promotion of wound healing (36–38).

b. Adeno-associated viruses. Adeno-associated viruses (AAVs) were once considered poor vectors due to their limiting packaging size (about 4.7 kb). However, the requirement for a helper virus (adenovirus or herpesvirus) for productive infection makes them one of the safest viral vectors (39). Currently, recombinant AAVs have a high safety profile because 96% of the AAV genome has been removed. AAVs can transduce a great variety of human cell types, both in vitro and in vivo (40). AAVs can exist in both integrated and non integrated forms, and are able to transduce replicating and nonreplicating cells (25,41). AAVs do not induce a strong innate immunologic or a cytotoxic T cell response, and long-term expression of the transgene is possible (42). However, they induce an antibody response, and the transfection efficiencies are usually low (42).

c. Retroviruses. These are the most widely used viruses for gene delivery. They are capable of delivering genes to

Table 1 Viral Gene Transfer Vectors for Cutaneous Gene Therapy

Vector Type	Advantages	Disadvantages
Retroviruses	Transduce almost all dividing mammalian cells; high efficiency of transduction; long-term expression of therapeutic gene	Random integration into host genome; do not transduce non-dividing cells
Lentiviruses	Infect dividing and non-dividing cells; high efficiency of transduction; long-term expression of therapeutic gene	Random integration into host genome
Adenoviruses	Low risk of serious side effects; high cargo capacity; high titers easily achieved; no insertion into host genome; infect dividing and non-dividing cells	Highly antigenic; transient expression of therapeutic gene; does nor integrate into host genome
Adeno-associated viruses	High safety profile; transduce almost all mammalian cell types; infect dividing and non-dividing cells	Low cargo capacity; transfection efficiency usually low; insert into host DNA; induce antibody response

nearly any dividing mammalian cell type and integrate their genomes onto host cell chromosomes (43). With the introduction of pantropic envelope proteins, ''pseudotyped'' retroviruses have been used to stably deliver genes to a much broader host range, including insect cells and nonmammalian eukaryotes (44). Retroviral vectors can accommodate between 8 and 9 kb of foreign DNA, and high titer virus production is easily obtained (25). Their transduction capacity is restricted to nonproliferating cells; therefore, their use in skin gene therapy has been limited to ex vivo approaches (19 and references therein, 25).

One of the most significant advances in retroviral vector applications is the use of lentiviruses. Lentiviruses have the unique capacity to infect nondividing, as well as dividing cells (45). This is of particular importance for gene therapy approaches in which target cells are nondividing or slow dividing, such as epidermal stem cells, epidermal basal cells, or nervous tissue cells (46–49). In addition, in vivo approaches have been tested with success in both skin and nervous tissue, and stable, long-term production of proteins in a variety of model organs, such as brain, optic nerves, and skin, has been observed for genes delivered by lentiviral vectors (46–50).

2. Nonviral Gene Transfer Vectors

Although viral vectors are quite efficient for gene delivery, safety concerns have led to the development of other means for gene transfer, the nonviral vectors. The use of nonviral vectors avoids some of the problems that occur with viral vectors, such as endogenous virus recombination, potential oncogenicity, and induction of an immune response (51). Overall, nonviral vector techniques for gene transfer can be classified into 2 groups: delivery by a chemical/biochemical vector or delivery of genetic material by physical methods (51,52).

a. Chemical or Biochemical Nonviral Vectors. The advantages of chemical or biochemical nonviral vectors include their lack of immunogenicity, the absence of a theoretical size limit for the therapeutic gene, the use of one transfer agent for any desired gene, and the consequently low cost (Table 2). The 2 main nonviral vectors include cationic polymers or proteins (e.g. dendrimers, polylysine, DEAE-dextran, protamine or polyethyleneimine), and cationic lipids (51–55). These reagents can be used with naked DNA, RNA-DNA oligonucleotides, ribozymes, or other type of genetic material to increase their uptake. In addition, other means of gene delivery for cutaneous gene transfer have been explored in recent years. An engineered transposase originally isolated from fish binds to the inverted repeats of salmonoid transposons in a substrate-specific manner and mediates the precise cut-and-paste transposition in fish, as well as in mouse and human cells (56). This is an active transposon system that can be used for the introduction of a therapeutic gene. In addition, the Φ31 bacteriophage integrase (57), which is a site-specific recombinase that stably integrates large DNA molecules containing an *attB* phage attachment-site sequence into genomic ''pseudo-attp sites,'' has been recently used in studies targeting epidermal progenitor cells (58). By implementing a Φ31 integrase-based gene transfer system, Ortiz-Urda et al. were able to stably transfer the type VII collagen alpha-1 (COL7a1) cDNA into the genome of primary epidermal progenitor cells of recessive dystrophic epidermolysis bullosa (RDEB) patients and to achieve phenotypic correction in skin regenerated using these cells. Clearly, this system is advantageous because it lacks the biosafety risks of viral vectors, it is more efficient than the stable transfer achieved with plasmid-based systems, and it allows for the stable integration of large DNA sequences into chromosomal DNA.

In the mean time, antisense DNA oligonucleotides (AS-ODNs), short-length synthetic DNA molecules that hybridize with specific mRNA sequences and silence gene expression, have been used in cutaneous genetic transfer studies (59–62). RNA-DNA oligonucleotides have been used for targeted single-base gene correction through homologous recombination and mismatch DNA repair (63–66). RNA ribozymes have been used to cleave mutant target RNA and to repair mutant RNA through splicing (67–69). Finally, small interfering RNAs (siRNA) are short double-stranded RNA molecules that are capable of binding and inducing the degradation of specific mRNAs (70,71). This relatively new discovered mechanism of sequence-specific gene silencing known as RNA interference (RNAi), has been applied successfully in mammalian cells (70,71), and its potential uses in cutaneous gene therapy remain to be fully explored.

b. Physical Methods. In recent years, many physical techniques for cutaneous gene transfer have been developed (Table 3). Direct intradermal injection of naked DNA has been shown to be an efficient mechanism for gene delivery to the skin. Using this technique, gene expression has been detected in both epidermal and dermal cells (2,47,72,73). The advantages of this method are its simplicity and safety, but it is relatively inefficient and coverage area tends to be small (2,51). Therefore, additional physical methods have been tested in order to improve the efficiency of in vivo gene transfer. For example, topical application of antisense oligonucleotides and of DNA complexed with liposomes is effective in targeting the epidermal cells (74–76). The use of epidermal enzymes, depilatory creams or the stripping of the skin with tape to induce structural disruption of the stratum corneum, can potentially increase the uptake of liposomal particles or oligonucleotides (19 and references therein). Electroporation, which is the use of electrical pulse fields to increase cell permeabilization (77), has been determined to increase gene uptake and transgene expression following injection of naked DNA in numerous tissues, including the skin (48,78–82). Puncture-mediated gene transfer, a method in which a device with a constant high-frequency oscillating bundle of fine metal needles, leads to DNA transfer and subsequent detection of reporter genes in skin cells (83). Particle-mediated gene transfer or ''gene gun'' is also an effective method to deliver genetic material to skin cells, producing high transgene expression. In this method, small DNA-coated particles are accelerated into tissues and are capable of penetrating the cell in virtue of their small size; once inside the cell, the DNA dissociates and its expression ensues (51,84,85). The major

Table 2 Chemical/Biochemical Vectors for Cutaneous Gene Transfer

Vector Type	Advantages	Disadvantages
Cationic polymers/proteins Cationic lipids	Lack of immunogenicity; no theoretical size limit for cargo capacity; can be used with naked DNA, RNA-DNA oligonucleotides, ribozymes or other genetic materials; low cost	Gene transfer less efficient than viral vectors; short-lived therapeutic gene expression; long term selection usually needed
"Naked" DNA	Lack of immunogenicity; low biosafety risk	Low efficiency of gene transfer; short-lived therapeutic gene expression
ϕ 31 bacteriophage integrase Engineered transposases	Precise integration of large DNA molecules into host genome; low biosafety risk; more efficient for stable gene transfer than plasmid-based systems; no theoretical size limit for cargo capacity	Further development needed for clinical applications

limitation of the gene gun technique is the degree of penetration into tissues, but it has been demonstrated that epidermal and dermal penetration is efficient to induce high levels of transgene expression in the targeted areas (86,87). In addition, other techniques such as ultrasound and hydrodynamic injection are being developed, but their current value for gene transfer to the skin is minimal (51).

IV. CANDIDATE DISEASES FOR CUTANEOUS GENE THERAPY

Many skin diseases are characterized by single gene mutations, either dominant or recessive, and can therefore possibly be corrected by destruction of the mutant gene product or introduction of a wild-type gene product. In addition to being used to correct inherited skin diseases, the skin can be used as a delivery system to secrete various polypeptides, such as enzymes, growth factors, and cytokines, into the systemic circulation. Furthermore, taking advantage of the potent antigen-presenting dendritic cells in the epidermis (Langerhans cells) and dermis, the skin can be used as a route of immunization against tumor-associated or infectious-associated antigens.

Table 3 Physical Methods for Cutaneous Gene Transfer

Technique
Direct intradermal injection
Topical application
Particle-mediated gene transfer ("gene gun")
Puncture-mediated gene transfer
Electroporation
Ultrasound[a]
Hydrodynamic injection[a]

[a]Current value for cutaneous gene transfer is minimal.

A. Cutaneous Gene Therapy for Inherited Skin Diseases

1. Characterization of Disease Genes

The molecular basis of a number of inherited skin diseases has been elucidated in recent years. These include genetic lesions leading to skin blistering, abnormal cutaneous cornification, and predisposition for cancer. In the epidermis, mutations in the genes expressed in the basal keratinocytes usually lead to skin fragility and blistering. Examples include, K5 and K14 mutations in epidermolysis bullosa (EB) simplex (88,89), laminin 5 mutations in a subset of junctional EB (90,91), and defects in type VII collagen in dystrophic EB (92,93).

Mutations in genes expressed in the suprabasal keratinocytes lead to abnormal epidermal terminal differentiation and are often manifested as keratinization disorders known as ichthyoses, characterized by thickened and scaly skin (94). Examples include transglutaminase 1 (TGase1) mutations in lamellar ichthyosis (LI) (95), and K1 and K10 mutations in epidermolytic hyperkeratosis (EHK) (96–98). Genetic defects in genes expressed in the skin have also been linked to cancer predisposition, as in the cases of patched mutations in basal cell nevus syndrome (99,100) and mutations in XP genes in xeroderma pigmentosa (101).

Understanding of the molecular basis of disease pathogenesis provides the cornerstone in designing rational gene therapy strategies. Correction of recessive phenotypes, in principle, requires the correct expression of the wild-type gene product where it was previously absent or defective. Much progress has been made in such attempts and success has been recently achieved in model systems (20–22,102). Correction of dominant-negative phenotypes, however, presents more of a challenge. The dominant-negative gene product has to be efficiently disrupted before the normal functions of the wild-type gene product can be restored. Several strategies are under development to circumvent the negative effects of mutant gene products.

2. Establishment of Disease Models

On understanding the mechanisms of a particular skin disease, an appropriate model has to be established to test possible gene therapy strategies before they are applied to patients. Such a model can be either a human tissue/animal chimeric model or an entirely animal model.

For recessive diseases, the pathologic phenotypes develop only when both alleles are mutated or deleted from the genome. Although mutant cells retaining disease characteristics from patients can be used to test therapeutic approaches in vitro or grafted onto an animal (103), such material is often limited. The development of mouse embryonic stem (ES) cell techniques and the use of homologous recombination made it possible to obtain virtually unlimited material if a mouse model could be generated for a particular disease. The ES cell techniques and homologous recombination allow manipulation of the mouse genome in a finely controlled manner, where mutations can be made in a particular gene to observe the consequence of its alteration during development and differentiation. Mouse models for several recessive skin diseases have been made by making a null mutation in epidermal genes, such as TGase 1 gene in LI (104) and type VII collagen gene in dystrophic EB (105). These mouse models not only provided much information on the mechanisms of disease pathologic study, but are also invaluable model systems to test gene therapy approaches for these diseases.

For dominant diseases caused by haploinsufficiency, as in the case of striate palmoplantar keratoderma (106,107), introduction of a second wild-type allele for desmoplakin may eliminate the disease phenotype, as in the case for recessive diseases. For diseases caused by a dominant-negative mutation, traditional transgenic mouse models were very informative in helping us to understand the disease mechanisms. In this approach, a mutated transgene is introduced into the mouse genome, and when it is expressed at a high enough level to compete with gene products from both wild-type alleles, a phenotype is observed. Mouse models were made in this manner for epidermolysis bullosa simplex (EBS) (108) and EHK (109). However, such mouse models cannot be used to test gene therapy approaches. First, because both wild-type alleles are present, the mutant gene product has to compete with wild-type product from both alleles. Second, the transgene is integrated into the genome randomly; therefore, its expression level and consequently the severity of the phenotype are affected by the surrounding sequences. Thus, the ratio of wild-type to mutant gene product, a crucial factor in judging the success of the therapy, remains variable in these models.

We took advantage of the mouse ES cell techniques and homologous recombination, and generated several mouse models for dominant skin diseases. ''Hot spot'' mutations in the 1A region of the rod domain in K14 and K10 have been linked to EBS and EHK, respectively (110,111). Using a knock-in/replacement strategy, we replaced a wild-type K14 or K10 allele with a mutant allele containing a ''hot spot'' mutation (112,113). Heterozygous mutant mice developed phenotypes similar to EBS and EHK, respectively, as expected for the dominant mutations. These mouse models mimic the diseases at both the genetic and phenotypic level, and will be the ideal systems to test gene therapy approaches for EBS and EHK.

3. Progress in Gene Therapy of Specific Skin Disorders

a. Lamellar Ichthyosis. The autosomal recessive ichthyoses are a clinically heterogeneous family of diseases characterized by abnormal cornification, and comprise LI and congenital ichthyosiform erythroderma (114). In LI, patients are born encased in a ''collodian'' membrane that is later shed and followed by development of large, thick scales of varying degrees of erythema. Palmar and plantar hyperkeratoses are often present.

Defects in the gene encoding keratinocyte TGase 1 were identified in a number of patients with LI (95,115). TGase 1 is normally expressed in differentiated keratinocytes and catalyzes cross-linking of cornified envelope precursor molecules, such as involucrin, loricrin, and small proline-rich proteins (116,117). With loss of TGase 1 function in the formation of insoluble cornified envelope, the barrier function of the outer epidermis is disrupted (94,118). Therefore, restoration of the TGase 1 enzymatic activity may represent a possible means of correcting the LI disorder.

Correction of recessive phenotypes requires the introduction of a wild-type gene product. A high-efficiency retroviral vector containing a wild-type TGase 1 gene was used to transduce mutant keratinocytes form LI (21). More than 98% of the primary cells expressed wild-type TGase 1, as measured by the proportion of keratinocytes positive for the transferred gene compared with the total number of cells determined by propidium iodide counterstaining (21). TGase 1 enzymatic activity was restored to normal levels and was targeted to the membrane fraction. In addition, transduced keratinocytes also demonstrated restored involucrin cross-linking and normal cornification (21). When these transduced keratinocytes were grafted to immunodeficient mice, the regenerated skin displayed restored TGase 1 protein expression in vivo and was normalized at the levels of histology, clinical surface appearance, and barrier function (20). However, TGase 1 expression in the human skin graft was lost in a month because of silencing of the vector (22).

Besides transplantation of genetically modified cells, another way to deliver genes to the skin is through direct administration to the intact tissue. Choate and Khavari (119) regenerated skin from patients with LI on nude mice to examine the corrective impact of direct injection of naked plasmid DNA. Regenerated LI skin received repeated in vivo injections with a TGase 1 expression plasmid, and restoration of TGase 1 expression in the correct tissue location in the suprabasal epidermis was observed. However, unlike LI skin regenerated from keratinocytes first transduced in vitro with a retrovirus carrying TGase 1 prior to grafting, directly injected LI skin displayed a nonuniform TGase 1 expression pattern (119). In addition, direct injection failed to correct the central histologic and functional abnormalities of LI. These results show that

partial restoration of gene expression can be achieved through direct injection of naked DNA into the human skin diseased area, but underscore the need for new advances to achieve efficient and sustained plasmid-based gene delivery to the skin.

b. Junctional EB. There are 2 major forms of junctional EB, the Herlitz variant or EB letalis, and generalized atrophic benign EB (GABEB). In addition, there are several other variants (120). Both forms are transmitted in an autosomal recessive manner, and both have onset at birth and are associated with marked skin fragility and generalized blister formation. Although scarring and milia formation are usually absent in both forms of junctional EB, skin atrophy is a characteristic finding in GABEB patients who also tend to have marked dystrophic or absent nails, palmoplantar hyperkeratosis, and significant scarring alopecia of the scalp (120). A characteristic feature of Herlitz disease is the development of large, nonhealing areas of granulation tissue; common sites include the perioral and perinasal areas, trunk, and nape of the neck. In addition, extracutaneous involvement may occur in both forms of junctional EB. In Herlitz disease, findings may include oral blisters and erosions, dysplastic teeth, marked growth retardation, and severe anemia. In contrast, patients with GABEB have milder mucosal involvement (oral cavity, conjunctiva, and esophagus) and early loss of permanent teeth, but neither growth retardation nor anemia (120). Unlike dystrophic EB, musculoskeletal abnormalities are absent in both forms of junctional EB.

Junctional EB involves dissociation of the dermal-epidermal junction, which occurs beneath the basal cell layer, but above the lamina densa. On examination by electron microscopy, junctional EB is caused by dissolution of the lamina lucida. Mutations in a number of genes encoding vital structural proteins, including laminin 5 components $\alpha 3$, $\beta 3$, $\gamma 2$ (90,91), BP180 (type XVII collagen or BPAG2) (121), integrin $\beta 4$ (122), and plectin (123) have been identified in junctional EB.

Several somatic gene therapy approaches have been tested to correct the junctional EB phenotype. In one study, when keratinocytes from a patient with Herlitz junctional EB with a mutation in laminin 5 $\beta 3$ were transduced with a $\beta 3$ transgene, the transduced keratinocytes synthesized $\beta 3$ peptide that assembled with the endogenous $\alpha 3$ and $\gamma 2$. They assembled into biologically active laminin 5, which was secreted, processed, and deposited into the extracellular matrix. Reexpression of laminin 5 induced cell spreading, nucleation of semidesmosomal-like structures, and enhanced adhesion to culture substrate (124). Organotypic cultures with the transduced keratinocytes reconstituted epidermis closely adhering to the mesenchyme and presenting mature hemidesmosomes, bridging the cytoplasmic intermediate filaments of the basal cells to the anchoring filaments of the BM (124).

A mouse line with targeted disruption of laminin $\alpha 3$ (LAMA3) (laminin 5 $\alpha 3$) was recently created (125). Although the mutation in homozygous pups caused neonatal lethality, cells isolated from these pups could be used to test therapeutic approaches in vitro. In a study carried out to restore BP180 function in cultured junctional EB patient keratinocytes and skin graft through gene transduction (126), the transduced cells had normalization of their adhesion parameter. In addition, a revertant mosaicism was reported recently in a patient with GABEB, representing a ''natural gene therapy'' (127). Importantly, reversion of the affected genotype to carrier (heterozygote in a recessive disease) genotype in about 50% of the keratinocytes was sufficient for the normal functioning of the skin.

A risk for reintroducing a therapeutic wild-type protein into patients with a recessive disease is that immunologic responses may be elicited. Extra caution has to be taken to decrease such possibility.

c. Epidermolysis Bullosa Simplex. Epidermolysis bullosa (EB) is a group of hereditary mechanobullous disorders with at least 11 distinct forms, 7 of which are dominantly inherited. The EBS subtype is characterized by intraepidermal blistering, and most cases are due to dominant keratin mutations. The estimated incidence for EBS is 10 per 1 million births in the United States (128), with a considerable perinatal mortality rate because of electrolyte imbalance, marked protein loss and sepsis. The most severe form of EBS, epidermolysis bullosa herpetiformis (EB herpetiformis) or Dowling-Meara variant (EBS-DM), presents at birth with generalized blistering (129). Blisters occur characteristically in groups (herpetiform) on the trunk and extremities, including palms and soles, and usually heal without scarring. Development of hyperkeratoses starts later in childhood.

Approximately 70% of the reported mutations in EBS-DM occur at the same mutational ''hot spot,'' codon 125, an arginine located at the beginning of the rod domain of K14. On ultrastructural examination, EBS-DM cells have perinuclear aggregates of keratin filaments in the basal cells instead of keratin bundles throughout the cytoplasm in normal basal cells (128). The weakened basal cells rupture on mild mechanical trauma, and the clinical phenotype is blistering within the basal cell layer. Because K14 is only expressed in the basal keratinocytes, the suprabasal cells appear normal and undergo normal terminal differentiation.

Two mouse models were previously developed, including a transgenic model (108) and a K14 null model (130). Although both models helped us to understand the disease mechanisms, and the K14 null model also provided important insight into K15 functions, neither mimics EBS-DM at the genetic level and therefore cannot be used to test gene therapy approaches for the diseases. We recently developed a mouse model for EBS-DM, where a wild-type K14 allele was replaced with a mutant allele in the mouse germline (112). The presence of a neomycin-resistance cassette in an intron affected expression from the mutant K14 allele, and the heterozygotes had subclinical phenotypes. But homozygous pups developed extensive blisters and died shortly after birth. When the neo selection cassette was removed by Cre-mediated excision, the resulting heterozygous pups developed large blisters, as was expected for this dominant mutation. To our knowledge, this is the first mouse model that mimics EBS-DM at both the genetic and phenotypic level. Unfortunately, the pups died because of severe blistering.

To overcome such problems, we recently developed a transgenic mouse model that allows the focal deletion of geno-

mic sequence via Cre-mediated excision. A transgenic mouse line carries a Cre recombinase fused to a truncated progesterone receptor, driven by a K14 promoter (131). These mice were crossed with mice heterozygous for the mutant K14 allele with the neo cassette flanked by loxP sites, and heterozygous mutant mice carrying the transgene were obtained. On topical treatment with an antiprogestin, the neo cassette can be deleted in focal areas by activated Cre. We expect that in such areas the mutant K14 expression would be comparable with that of the wild-type K14, therefore causing blistering in the skin. We were indeed able to induce blisters using this system, and the mice remained viable (112). This transgenic mouse model is ideally suited to test gene therapy approaches for EBS-DM. In this mouse model, the mutant K14 allele can be focally activated in epidermal stem cells, and following topical administration of an inducer, blisters develop in treated areas. However, after a few weeks, blisters heal and never reappear. Some skin disorders are characterized by a mosaic pattern, with alternating stripes of affected and unaffected skin that follow the lines of Blaschko. These nonrandom patterns are believed to be caused by postzygotic mutations that occur during embryogenesis. Interestingly, a mosaic form of EBS has never been reported. It has been suggested that basal stem cells carrying a postzygotic mutation in K5 or K14 would have a selective disadvantage and be rapidly displaced by wild-type basal stem cells, which can move laterally. Using laser capture microdissection, we have shown that the induced blisters healed by migration of surrounding nonphenotypic stem cells into the wound bed. Thus, our model predicts that if EBS stem cells could be corrected, they will have a selective growth advantage when introduced into areas prone to blistering. This observation provides an explanation for the lack of mosaic forms of EBS-DM. In addition, it has important implications for gene therapy because it predicts that defective EBS stem cells will be replaced by nondefective stem cells. Another unexpected observation from this mouse model was the discovery that mice that express the mutant K14 allele at levels approximately 50% of wild-type K14 do not exhibit a skin phenotype. Previously, it had been assumed that gene therapy approaches for dominant disorders like EBS must aim to either correct the mutant allele, or completely inhibit its expression. Our model predicts that the EBS phenotype may be eliminated by overexpression of the normal K14 allele or partial suppression of the mutant K14 allele, thus increasing the ratio of wild-type to mutant protein. Technically, it is much easier to design gene therapy strategies that would achieve a partial suppression of a mutant dominant allele rather than a complete correction or suppression. Therefore, we will try to correct the EBS-DM phenotype by altering the ratio of wild-type to mutant K14 by increasing expression of wild type K14. This will be done using a viral vector expressing wild-type K14. In addition as an alternative strategy to correct the EBS-DM phenotype, we will transduce EBS-DM keratinocytes with a viral vector expressing a ribozyme designed to specifically degrade mutant K14 transcripts.

d. Epidermolytic Hyperkeratosis. This disease, also called bullous congenital ichthyosiform erythroderma, is inherited in an autosomal dominant mode, with an incidence of 1 in 200,000 to 300,000 newborns (128). Up to 50% of the reported cases arise sporadically. Affected children present at birth with erythroderma, blistering, and peeling. Erythroderma and blistering diminish during the first year of life, and hyperkeratoses develop, predominantly over the flexural areas of the extremities. On histopathologic examination, findings consist of hyperkeratosis and parakeratosis, lysis of the suprabasal keratinocytes, and perinuclear vacuolar degeneration. The basal keratinocytes appear normal but exhibit hyperproliferation (129). The transit time for keratinocytes to move from the basal layer to the stratum corneum is remarkably shortened and takes only 4 days in EHK patients instead of the normal 4 weeks (132). Mutations to K1 and K10 have been linked to EHK, and a mutational ''hot spot'' at Arg 156 in K10 has been identified in most severe cases of EHK (98,133). Three mouse models were previously generated, including 2 transgenic models (109,134) and a K10 null model (135). However, because they do not mimic EHK at the genetic level, none can be used as a model system to test gene therapy for this disease.

We recently generated a mouse model that mimics EHK at both the genetic and phenotypic levels by replacing a wild-type K10 allele with one that has a point mutation at Arg 156 (113). We will attempt correction of the phenotype by altering the ratio of wild-type to mutant K10 by increasing expression of wild-type K10. To do this, we will collect epidermal stem cells from heterozygous mutant mice, and transduce them with viral vectors (both retroviral and lentiviral vectors) to overexpress the wild-type K10 allele. In addition, we will attempt to correct the EHK phenotype by suppressing the expression of the mutant K10 allele by transducing EHK cells with a viral vector expressing a ribozyme specifically designed to cleave the mutant K10 transcripts. As opposed to EBS, in which a correction of the mutation in the basal keratins translates into a growth advantage, corrected and mutant stem cells in EHK will proliferate at similar rates because the mutant K10 is not expressed in stem cells, but only in the progeny of stem cells after they have differentiated and moved into the suprabasal layers. There is no selection against epidermal stem cells with K10 mutations, and these stem cells continue to give rise to defective differentiated progeny. It has recently been demonstrated that topical selection with colchicine can be used to select, in vivo, for human keratinocyte stem cells transduced with the multidrug-resistance gene (MDR) (136). Therefore, to efficiently amplify the corrected cell population, we will include the MDR as a selection marker in our targeting strategy to ablate defective epidermal stem cells and allow their replacement with corrected stem cells, which are expected to repopulate the epidermis.

e. X-linked Ichthyosis. X-linked ichthyosis (XLI) is caused by a deficiency in steroid sulfatase (STS), which leads to the accumulation of cholesterol sulphate and results in abnormal scaling skin (137). It is inherited in a pseudoautosomal mode, escaping X inactivation in both humans and mice. Transduction using a retroviral expression vector in vitro with

the STS gene leads to restoration of STS protein expression as well as enzymatic activity (102). In the same study, it was shown that transduced XLI keratinocytes from XLI patients regenerated epidermis histologically indistinguishable from that formed by keratinocytes from patients with normal skin, after grafting onto immunodeficient mice. In addition, the transduced XLI epidermis presented a return of barrier function parameters to normal.

f. Xeroderma Pigmentosa. These are a group of autosomal recessive disorders associated with defects in a number of DNA repair genes, and are characterized by inadequacies in DNA repair after UV injury (101,138). XP genes that are involved in a specific mechanism of DNA repair called nucleotide excision repair, fall into 7 complementation groups (XP-A to XP-G), and all have been cloned (139). Xeroderma pigmentosa (XP) patients have increased susceptibility to epidermal neoplasms, such as basal and squamous cell carcinomas, and malignant melanoma (140). XP affects both the basal and suprabasal compartments, including melanocytes. Because every cell that is unable to repair the UV-induced DNA damage should be considered a potentially tumoral cell, any treatment to be curative, must target every cell in the epidermis, including every stem cell and melanocytes. Although promising attempts to correct the genetic defects in this condition have been performed (141–143), XP represents a challenging condition for cutaneous targeted gene therapy because high-efficiency targeting of melanocytes remains to be developed.

g. Other Conditions. In addition to the dominant-negative mutations in keratins that cause various blistering diseases and hyperkeratoses, several other diseases are inherited in a dominant mode through haploinsufficiency, such as palmoplantar keratoderma that results from desmoplakin mutations (106,107). Gene therapy strategies could involve introduction of a wild-type allele, and host immunologic reactions will not be a concern in this case.

B. Cutaneous Gene Therapy for Systemic Diseases: The Skin as a Bioreactor

Epidermal keratinocytes secrete a variety of proteins such as collagen VII, laminin, proteinases, proteinase inhibitors, growth factors, and cytokines (144 and references therein). Studies using cultured human keratinocytes, grafted onto athymic mice and rats, demonstrated that keratinocytes secrete proteins that reach the systemic circulation. One of the first studies of the secretory function of keratinocytes was carried out by Lorne Taichman and colleagues. They first monitored secretion of human apolipoprotein E (apoE) in cultured human keratinocytes (145). The protein was identified as apoE on the basis of molecular weight, isoform pattern, and immunoreactivity. When they grafted human keratinocytes onto athymic mice and rats, human apoE was detected in the systemic circulation of graft-bearing animals as long as the graft remained on the animals (146). Within 24 h of graft removal, human apoE was not detected in the plasma, indicating that human apoE in the plasma resulted from continuous production of the protein by grafted human keratinocytes. These results showed that proteins as large as apoE (299 amino acids) can transverse the epidermal-dermal barrier and achieve systemic circulation.

Once it was established that epidermis-secreted proteins could reach the central circulation, genetically modified keratinocytes were used to test whether they could deliver transgene products into the bloodstream. Subsequent experiments using both in vivo and ex vivo approaches have been successful in delivering different polypeptides, such as growth hormone, erythropoietin (Epo), factor VIII and IX, leptin, and interleukin 10 (IL-10), to the circulation. In our laboratory, we have further enhanced the usefulness of the skin as a bioreactor by developing a bigenic gene switch system that allows focal induction of transgene expression via topical administration of an inducer (147). Therefore, because of its ability to deliver various polypeptides into the systemic circulation, its accessibility and abundant vascularization, added to the gene switch system development, the skin is a very attractive tissue to test gene therapy strategies for systemic conditions that respond to delivery of polypeptides into the circulation.

1. Progress in Cutaneous Gene Therapy of Specific Systemic Disorders

a. Hemophilia A. This is an X-linked inherited disease caused by deficiency of factor VIII and has an incidence of 1 in 5000 male live births (148). This condition occurs in mild, moderate, and severe forms, reflecting the mutational heterogeneity seen in the factor VIII gene, and symptoms vary from excessive bleeding only after trauma or surgery to frequent episodes of spontaneous or excessive bleeding after minor trauma, particularly into joints and muscles (148–149). In a recent study, factor VIII-deficient transgenic mice expressing human factor VIII under the control of the involucrin promoter were generated (150). Plasma factor VIII activity and correction of the phenotype were seen in this mouse model. In the same study, skin explants from these transgenic mice were grafted into factor VIII double knockouts, which showed plasma factor VIII activity of 4% to 20% normal and had improved whole blood clotting (150).

b. Leptin Deficiency. Leptin, a 16-kDa protein hormone, is involved in the regulation of body weight in mammals (151,152). It is secreted primarily by adipocytes, and it has been shown to regulate food intake and neuroendrocrine function through its action in the hypothalamus (152). In accordance with the phenotype seen in the *ob*/*ob* mice (153), it has been determined that congenital leptin deficiency is associated with early-onset obesity in humans (154). Leptin replacement therapy has provided encouraging results in clinical studies (155); however, the need for repetitive dosing has prompted an alternative approach using gene therapy to correct this condition. In a recent study, a cutaneous gene therapy approach for leptin deficiency was successful in correcting the mouse *ob*/*ob* phenotype (156). Here, immunodeficient *ob*/*ob* mice grafted with skin implants from mice overexpressing leptin, reached body weight equivalent to that of wild-type animals. In addition, immunosupressed *ob*/*ob* mice that were trans-

planted with skin grafts made of human keratinocytes transduced with a leptin cDNA-carrying retroviral vector, showed weight reduction concomitant with a decrease in blood glucose and food intake (156).

c. Anemia due to Erythropoietin Deficiency. Epo is a kidney-produced glycoprotein that regulates red cell production. It binds to its receptor found in erythroid progenitor cells, activating a signaling pathway that leads to the increase of survival of these cells by inhibiting apoptosis (157). In 1987, recombinant human Epo (rHuEpo) was approved in the United States for the treatment of anemia of end-stage renal disease (157). In addition, its therapeutic use has been extended to other conditions, such as anemia associated with Zidovudine treatment of patients with AIDS, anemia secondary to chemotherapy in the treatment of cancer, anemia of pregnancy, anemia of prematurity, myelodysplastic syndrome, and bone marrow transplantation (157 and references therein). In a recent study, a lentiviral vector encoding HuEpo was delivered by single intracutaneous injection into human skin grafts on immune-deficient mice (158). The investigators demonstrated that HuEpo was present in serum, and its levels increased in a dose-dependent fashion. In addition, the hematocrit improved within 1 month after lentiviral injection and remained stable for almost 1 year.

d. Contact Hypersensitivity. Contact hypersensitivity (CHS) responses are regulated by T cells that release cytokines and attract other inflammatory cells after reacting with antigen. IL-10 has been known to be a key regulatory cytokine in both inflammatory and immune responses. Studies have demonstrated that IL-10 is involved in the regulation of the hypersensitivity response because recombinant IL-10 (rIL-10) prevented the elicitation of CHS in previously sensitized mice (159). Meng et al. injected a DNA plasmid containing human IL-10 into the dorsal skin of hairless rats (160). They detected local expression of mRNA and protein in a dose-dependent manner. Further, they showed that the transduced keratinocytes produced and released IL-10 into the circulation by detecting it in the bloodstream, and by quantifying a reduced response to challenge in distant areas from the injection site of previously sensitized animals.

e. Growth Hormone Deficiency. Original experiments of the release of an exogenous growth hormone by transduced keratinocytes into the circulation were performed by Morgan et al. (161). Using the recently developed bigenic gene switch mouse model, we showed that after a single induction, high levels of the therapeutic protein, human growth hormone (hGH), were released from keratinocytes into the circulation (147). The serum levels of hGH were dependent on the amount of inducer applied, and repeated induction resulted in increased weight gain by transgenic vs. control mice. Furthermore, physiological levels of hGH were detected in the serum of nude mice after topical induction of small transgenic skin grafts. These results clearly demonstrate the feasibility of using the gene switch system to regulate the delivery of growth hormone into the circulation for the treatment of growth hormone deficiency.

f. Other Conditions. Additional examples of the skin as a bioreactor approach and its potential use have been performed. For example, two forms of apoE, both the endogenous human apoE and a recombinant form from a transfected vector, were detected in the serum of athymic mice bearing grafts of modified human keratinocytes (162). When human keratinocytes in culture were transduced with a retroviral vector carrying factor IX gene, they secreted active factor IX into the medium (163). When they were grafted onto nude mice, small quantities of factor IX were detected in the bloodstream (163).

C. Other Applications of Cutaneous Gene Therapy

In addition, cutaneous gene therapy has also been applied to other physiopathological processes, such as wound healing, immunoregulation, and cancer.

1. Wound Healing

The process of wound healing involves 3 stages: inflammatory reaction, formation of granulation tissue, and tissue remodeling (164). All these events are known to be regulated by different cytokines and growth factors. There are obvious advantages of gene transfer techniques for treatment of wound healing abnormalities or to enhance the wound healing rate because the epidermal barrier is defective, only limited target gene expression is needed, and the treated area is usually localized (165). Tyrone et al. successfully treated ischemic dermal ulcers in rabbits by topical application of platelet-derived growth factor (PDGF)-A or-B-DNA plasmids embedded within a collagen latice. They showed that PDGF-A and PDGF-B DNA substantially increased the formation of new granulation tissue, epithelialization, and wound closure (166). Subcutaneously injected liposomal insulin-like growth factor 1 (IGF-1) cDNA construct was shown to effectively promote reepithelialization of burn wounds, by decreasing prolonged local inflammation through modulation of the expression of pro- and anti-inflammatory cytokines (167). Intradermal injection of an AAV vector expressing human vascular endothelial growth factor A (VEGF-A) to full thickness excisional wounds in rats was found to induce new vessel formation and enhance wound healing rate (168). Similar results were obtained by Romano Di Peppe et al. using topical application of an adenovirus vector to deliver VEGF on excisional wounds of streptozotocin-induced diabetic mice (169). In other studies, liposomal keratinocyte growth factor cDNA gene delivery to acute wounds in rats, enhanced wound healing by increasing cell proliferation, reepithelialization, and neovascularization, by reducing cell apoptosis, and by activating mesenchymal cells through the induction of IGF-1 expression (170). Other groups have tested different modalities of gene transfer to skin wounds, such as the gene gun approach and the use of genetically modified cultured skin substitutes, with promising results (171,172).

2. Cutaneous Immunomodulation and Cancer Gene Therapy

Gene transfer has become a practical method to induce an immune response. The skin is rich in antigen-presenting cells that are able to initiate and control a specific immune response. It has been demonstrated that injection of naked DNA that encodes antigenic epitopes can induce specific humoral immune responses (173). The advantages of immunomodulation by genetic vaccination are evident; for example, there is no need to isolate and purify protein for vaccination, and it circumvents the use of life or attenuated viral vaccines. In addition, cutaneous transfer of plasmid DNA or mRNA allows for the concurrent delivery of genetic material encoding antigenic epitopes and immunomodulators (174,175). All these make cutaneous genetic transfer a desirable method for engineering specific immune responses. This strategy has been used to induce immune responses with different objectives, such as antitumoral immunotherapy, treatment/prevention of infections, and treatment of autoimmune diseases (175). Cutaneous gene transfer studies that induce an immune response and hold promise as plausible options in the treatment of certain malignancies are briefly presented. The gene transfer by gene gun, and subsequent expression in the skin of different the antitumoral cytokines IL-2, IL-6, and tumor necrosis factor-α and-β (TNF-α and TNF-β), led to tumor regression and/or increased survival in mice with subcutaneously implanted tumors (176). Regression of established primary and metastatic murine tumors was documented after cutaneous gene gun delivery of a plasmid carrying the mouse IL-12 (177). In the same study, the researchers were able to demonstrate that a tumor-specific immunological response had been induced in the treated mice. In a clinical trial conducted by Thurner et al., dendritic cells were isolated, propagated ex vivo, and pulsed with a Mage-3A1 peptide. Subsequently, the cells were injected subcutaneously to advanced melanoma patients; regression of some metastases was seen in 6 of 11 patients, and expansion of specific cytotoxic T cells was seen in 8 of 11 patients (178).

3. Cutaneous Gene Therapy Approaches for Melanoma and Squamous Cell Carcinoma

The incidence of malignant melanoma (MM) has been on the rise in the last years. Squamous cell carcinoma (SCC) is a common malignancy of the epidermal keratinocytes. Due to the advantages that make the skin an amenable tissue for gene transfer, MM and SCC are frequently used as models for tumor-specific gene therapy. Different strategies have been applied for both conditions. For instance, adenoviral-mediated transfer of the herpes simplex thymidine kinase ''suicide'' gene (tk) by direct intratumoral injection, followed by ganciclovir administration, was used to treat human MM established in nude mice (179). In a different study using the B16 melanoma model, a synergistic effect was observed when combination therapy by adenovirus-mediated transfer of tk and IL-2 or tk and granulocyte-macrophage colony-stimulating factor was used (180). Similar approaches have been employed in studies with SCC models (181,182). Besides gene transfer mediated by adenoviral vectors, an in vivo liposomal-mediated approach with tk and mIL-2, and a transgenic model constitutively expressing a costimulatory molecule, have been performed for treatment of SCC (19 and references therein). In addition, other genetic transfer approaches have been employed in MM models, such as genetically altered fibroblasts and the use of helper virus-dependent, HSV-1 amplicon vectors on human MM xenografts (19 and references therein, 183).

V. FUTURE PERSPECTIVES

With the identification and characterization of the genetic basis and molecular biology of inherited skin and systemic diseases, added to the multiple advantages of the skin as a target organ for gene transfer, we are in a better position in designing feasible strategies for gene therapy using cutaneous gene transfer approaches. During the last decade, we have seen the development of highly efficient cutaneous gene transfer systems, and better animal models for both inherited skin and systemic diseases. Although the general idea is to introduce wild-type gene products or destroy mutant gene products (including the mutant gene itself), the patient-specific nature of some of the diseases will still be challenging in a case-specific manner. Clearly, to apply these advances to human clinical trials, more efficient gene transfer methods with increased and prolonged levels of expression, high safety profile, low cost, and ease of administration must be developed. Nonetheless, the recent advance of model systems, epidermal stem cell techniques, and gene delivery efficiency highlight the promise for successful gene therapy of conditions using cutaneous gene transfer approaches.

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33

Gene Therapy for Childhood Onset Blindness

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I. BACKGROUND: GENE THERAPY AND LEBER CONGENITAL AMAUROSIS

In recent years, mutations in a large number of different genes have been implicated in the pathophysiology of retinal degenerative diseases (RetNet, http://www.sph.uth.tmc.edu/RetNet). Among these are mutations that lead to particularly severe early onset blinding disease. With the recent developments of methods for in vivo retinal gene delivery [reviewed by (5,7,12,15)], it has become possible to evaluate efficacy of gene therapy in animal models for particular forms of these blinding diseases. We selected a canine model for study of efficacy of gene therapy for one such disease, Leber congenital amaurosis (LCA). LCA is usually diagnosed in infancy as it severely disables its subjects, leaving them with minimal visual function and abnormal ocular movements (nystagmus). There are at least half a dozen genes that, when mutated, can lead to LCA (11,31). One of these, *RPE65*, named for the evolutionarily conserved retinal pigment epithelium (RPE)-specific 65-kDa protein that it encodes (4,28), accounts for approximately 7% to 16% of these cases (22,24,26). Mutations in *RPE65* can also lead to other severe and early onset blinding diseases, including retinitis pigmentosa (RP) (14,26,31,38).

II. RESULTS OF INITIAL STUDIES IN THE *RPE65* MUTANT DOG

A. A Canine Model for LCA: A Unique Resource

An advantage of selecting the *RPE65* gene defect for study is the availability of a large animal model for the LCA disease: the *RPE65* mutant dog. This animal suffers from an autosomal recessive inheritance of a retinal disease with severe visual deficits but a relatively slow degenerative component (2,27,41). A homozygous 4-bp deletion in the canine RPE65 coding sequence (2,39) is responsible for the phenotype in this animal. The deletion results in a frame shift leading to a premature stop codon. More than two-thirds of the wild-type polypeptide is eliminated due to this premature stop.

The exact function of the RPE65 protein is unknown, but it is known that this protein plays an important role in vitamin A metabolism (23,32,35). Without RPE65 protein, there is a deficiency of 11-cis retinal in the retina. This accounts for the abnormal qualitative and quantitative measures of visual function that are apparent early in life.

Examination of retinas of dogs who are homozygous for the defect reveals prominent RPE inclusions and slightly abnormal rod photoreceptor morphology present within the first year of life (41). There is also a lack of immunohistochemically detectable RPE65 protein (2). As the dogs age, a slowly progressive photoreceptor degeneration becomes apparent. Photoreceptor degenerative changes and loss are readily detectable in older (~5-year-old) dogs (41).

B. Proof of Principle of Gene Therapy for Retinal Degeneration

Efficacy of gene therapy for inherited retinal degeneration may be efficiently demonstrated studying animal models. Proof of principle for such treatments has been demonstrated using multiple strategies, multiple viral vectors, and several small animal models [reviewed by (5,7,12,15); also see (20,21,25,34,40)]. Successful strategies include delivery of wild-type cDNA in the case of loss-of-function disease (8,16,17,37,40), delivery of ribozymes that specifically target the mutant mRNA in the case of gain-of-function disease (19,18), delivery of neurotrophic factors (20,21,25), which

preserve the integrity of the photoreceptors, and delivery of genes with antiapoptotic function (9) in order to limit programmed cell death. Rescue has been achieved with a number of different vectors, including adenovirus, adeno-associated virus (AAV), lentivirus, and gutted adenovirus.

Clinical endpoints in many of these studies have involved electrophysiological evidence of improvement in retinal function as assessed by electroretinograms (ERGs). These can be recorded in noninvasive fashion. Because the diseases are usually bilaterally symmetrical, interpretation of results benefits from use of the contralateral eye as control. Results from retinal function testing have been supplemented with histological/immunohistochemical data obtained in terminal studies. In many of these paradigms, rescue has been detected for months after treatment. The studies involving the *RPE65* mutant dog have added additional outcome measures. These measures provide evidence that the brain of the treated animals can recognize that visual function has been restored (1).

C. Treatment Strategy Resulting in Rescue of Vision in *RPE65* Mutant Dog

The experimental objective was to deliver wildtype copies of the disease–causing gene (*RPE65*) to cell populations primarily affected by the gene mutation (i.e., the RPE). The minimum number of affected animals were used and analyses are described only for those animals in which 1 eye received an adequate control treatment. Affected dogs were identified through molecular diagnosis and by performing baseline ERGs (1).

Unilateral subretinal injections of the corrective gene (the canine RPE65-encoding cDNA) carried by AAV (AAV.RPE65) were performed, thereby exposing the diseased cells to the virus. The virus was prepared using the genome and capsid of AAV serotype 2 (AAV-2/2). Injections were performed when the animals were 4 months of age. A constitutive promoter (chicken beta actin) was used, and the amount of virus delivered was $1–5 \times 10^{10}$ infective units of AAV. Approximately 20% to 30% of the retina was exposed to virus. The contralateral eyes received control injections of intravitreal AAV.RPE65. Intravitreal delivery of AAV-2 results in gene delivery to the inner retina but not the outer retina/RPE (13).

The animals were followed over time with regular clinical evaluations/laboratory testing to evaluate local/systemic toxicity. Evaluations included fundus exams, monitering of weight gain/loss, complete blood count (CBC), and blood chemistries (including albumin, globulin, alkaline phosphatase, bilirubin, blood urea nitrogen, calcium, chloride, creatinine, gamma-glutamyl transpeptidase, glucose, phosphorus, potassium, alanine aminotransferase, and aspartate aminotransferase). In eyes that received subretinal injections, retinas reattached within 24 h. There was minimal inflammation immediately after surgery. Injected portions of the retina could be reliably identified based on ophthalmoscopically detectable pigmentary changes at the level of the RPE (1).

Animals appeared healthy and active after treatment. CBCs (total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cells, and platelets), red blood cell characteristics (hemoglobin, mean corpuscular volume, and hemoglobin levels), and blood chemistries were within normal limits throughout the course of the study (1).

Immune studies (including humoral and cell-mediated responses to the vector and/or to the transgene product) were also performed on sera and anterior chamber fluid samples. There was no evidence of cellular immune response to either AAV or the transgene product. Antibodies directed against AAV-2 capsid proteins increased after intraocular delivery of AAV in serum but not in anterior chamber fluid. Antibodies directed against transgenic protein (RPE65) increased after intraocular delivery of the AAV.RPE65 virus) in serum but not in anterior chamber fluid. Interestingly, there was evidence of preexisting anti-AAV capsid antibodies in serum samples as the baseline humoral response levels were elevated. This did not prevent transduction (1).

Visual function testing was performed 3 to 4 months after treatment and included ERGs, pupillometry, measurement of visual evoked cortical potentials (VECPs) and measures of visual behavior (1).

D. Efficacy of Gene Therapy in *RPE65* Mutant Dog

In the *RPE65* mutant dog, rod photoreceptor-specific ERG waveforms are essentially flat from birth on, even using high-intensity stimuli. Quantitative testing of retinal function using ERGs 3 to 4 months after treatment revealed waveforms similar to those present in wild-type dogs, although reduced in amplitude (1).

VECPs were recorded in order to determine whether there was evidence of cortical vision. There were definitive VECP waveforms in the *RPE65* mutant dogs after subretinal delivery of AAV.RPE65 (1).

There was evidence of visual behavior in treated *RPE65* mutant dogs soon after treatment. Those animals could navigate an obstacle course by 4 months after treatment using their experimentally treated eyes (1). The animals also demonstrated preferential gaze by the treated eye. Figure 1 illustrates this preferential gaze by showing the tracking pattern of a dog treated with the experimental reagent in the right eye. The dog follows the person walking around him with his right (treated) eye. The dog turns his head sharply, when the person is outside his right visual field, so that the person can continue to be visualized by the dog's right (seeing) eye.

III. FUTURE STUDIES

A. Challenges in Planning a Gene Therapy Clinical Trial for LCA

In the studies described above, AAV-2-mediated delivery of a wild-type version of the defective *RPE65* gene was used to provide retinal function in a canine model of severe early

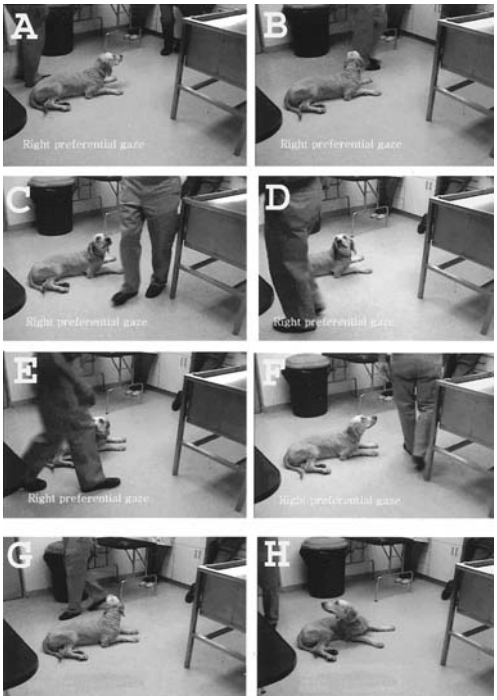

Figure 1 Frames in sequence from a video clip demonstrating right preferential gaze. Movie was filmed 9 months after subretinal delivery of AAV.RPE65 in the right eye in a mutant *RPE65* dog. The dog does not see the person when the person is behind him on his left (untreated) side (A). the dog follows the person walking around him with his right (treated) eye (B–H). When the person steps outside the visual field of the dog's right eye (H), the dog turns his head all the way to the right to visualize the person with his (seeing) eye.

onset retinal degeneration. There are many steps that must be taken before this treatment can be tested in humans with LCA. With the availability of both the canine and murine (*rpe65* knockout) (29,28) models for this disease, these steps can be taken using both animal models. The first and most important step is to establish safety of the treatment. Selection of regulatory elements that limit transgene expression to the target cells will minimize toxicity to other cells that might be exposed to the virus. Special attention must be paid to the possibility that the transgene could potentially be introduced to the brain via unwanted ganglion cell/optic nerve targeting (13).

The duration of the therapeutic effect should be characterized because LCA is a lifelong disease. The therapeutic window should be defined with respect to age of the animal/level of disease progression so that predictions can be made as to what the effects of treatment will be in younger and older humans with LCA.

The vector that provides the maximal therapeutic effect at minimum dose must then be selected. The recent demonstration that one can control cellular specificity, onset, and levels of transgene expression by packaging the AAV in capsids from AAVs of different serotypes may play a role in this process (3). Vector selection will be difficult. There is likely to be continued progress in developing viral vectors with slightly improved transduction qualities. At a certain point, a choice will have to be made: whether to select a particular vector as the candidate for formal toxicity studies (in anticipation of initiating a human clinical trial using this vector) or whether to perform additional efficacy tests on newly formulated vectors. The toxicity studies are rigorous, time-consuming, and expensive, and use a large number of animal subjects. It will be preferable not to have to repeat them using a second vector (unless the additional benefits of that vector warrant the effort). Once the ideal vector is selected, toxicity testing must be conducted to verify that the treatment does not cause any impairment to the eye or to the rest of the body. This must be performed using the same virus that is to be used in the proposed human clinical trial. Studies evaluating the possibility of readministration should also be performed with this vector in order to be able to predict whether it will be possible to treat the contralateral eyes of human patients at future timepoints.

B. Issues Regarding Future Patient Selection

While animal studies are in process, humans with LCA should be screened for their disease-causing mutations and individuals carrying homozygous/compound heterozygous *RPE65* disease-causing mutations should be identified. The typical progression pattern of the human disease (with respect to visual function measures and death of retinal cells) should be characterized. This will allow selection of the optimal noninvasive outcome measures for evaluation of effects of treatment in a human clinical trial. These outcome measures (likely measures of ERG/pupillometry response) will be quantitative and reproducible. One question that will arise is whether the first treatments, which will be phase I safety and toxicity tests, should be performed on adult vs. pediatric subjects. Also, an exit strategy should be planned if the results are unpleasant to the patient. Oliver Sacks describes, for example, the shock that a 55-year-old man had upon seeing for the first time since he was 6 years old, when he first opened his eyes after having had cataracts, present from childhood, removed (33). It should be emphasized, however, that the treatment will be deemed successful even if it is effective in a seemingly small way. If a patient can benefit from improved navigational skills that will expand his or her independence, this in itself will be a successful outcome. The ultimate goal will be, of course, to achieve vision that will allow the patient to lead as normal a life as possible.

C. Extrapolation of Success in Canine LCA Gene Therapy Studies to Other Forms of Early Onset Retinal Degeneration

A major challenge is how to extrapolate the successful treatment of the *RPE65* disease to treatment of other severe early

onset retinal degenerative diseases. One approach to meet this challenge is to identify the conditions that are responsible for the success of the *RPE65* dog study. Besides the virus delivery characteristics, there may be elements of the *RPE65*-caused disease that make it particularly amenable to treatment. Is the success due to the fact that the *RPE65* mutant retinal photoreceptors degenerate very slowly and thus there is a large therapeutic window during which the retina can be rescued? In some animal models (e.g., those with homozygous mutations in the beta subunit of rod cGMP phosphodiesterase) the degeneration is so rapid that rod photoreceptors do not even have a chance to fully differentiate before they are lost (10). In such diseases, it is likely that the treatment will have to be administered very early in life while the target cells are still viable (e.g., before postnatal day 10 in the rd1 mouse model of this disease) (8,17).

The AAV-2 vector may not have a rapid enough onset to make any impact in the disease progression of rapidly progressive degenerative diseases. One might want to employ a viral vector that results in a rapid onset of transgene expression. AAV-2 results in a slow (6–8-week) onset of transgene expression in the dog (6). In contrast, AAV-2 packaged with an AAV serotype 1 capsid (AAV-2/1) delivers transgenes that are expressed within a few days after injection in the mouse (3). If early administration with the optimal viral vector is not enough to achieve rescue, it may be possible to expand the therapeutic window by delivering the transgene at gestational timepoints. Recent progress in developing methods for in utero retinal gene transfer will expand these options (36). Finally, it will be desirable to test the rescue ability of the human *RPE65* cDNA (vs. the canine cDNA) in animal models before testing this cDNA in humans. The human *RPE65* cDNA was recently cloned and inserted into AAV vectors by N. Dejneka (personal communication; 2001) and promises to be useful in studies in both the *RPE65* mutant dog and the *rpe65-/-* mouse.

In particular diseases, there may be requirements of the disease model that will have to be met by developing alternative vectors/vector strategies. For example, one challenge is how to proceed with AAV if the cDNA and/or transgene cassette is too large to be packaged. The AAV cargo capacity is a maximum of 4.8 kb. In such a situation, one would have to consider other vectors that could carry the intact transgene cassette. Options include lentivirus, which can carry a cassette of $\sim$8 kb, and gutted adenovirus, which can carry a cassette of $>$30 kb. If one wanted to use AAV even though the transgene cassette was too large, one could harness the AAV dual vector approach and split the transgene construct into 2 complementary vectors, which would be trans-spliced in the target cell (30).

Another area that is likely to benefit from new technological developments is the ability to treat gain-of-function disease. Recent work involving gene correction strategies for both loss-of-function and gain-of-function disease and delivery of RNAi will likely be applied to animal models of early onset retinal degeneration using virus-mediated gene transfer.

IV. CONCLUSIONS

In summary, we have shown that efficacy of gene therapy for inherited retinal degeneration may be efficiently demonstrated studying animal models for the most clinically severe retinal degenerations (i.e., those that result in early onset blindness). AAV-2 was used to deliver corrective genes subretinally to a canine model of a severe retinal disease and efficacy of treatment was readily apparent. Success in this study may lead to identification of critical parameters for the success of gene therapy for early onset retinal degeneration and also to a human clinical trial designed to treat LCA.

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34

DNA Vaccines

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I. IMPORTANCE OF VACCINES

A. Historical Importance of Vaccines

Vaccination is a deliberate introduction of materials in humans to elicit immune protection against diseases (1). For example, some Indian Buddhists drank snake venom to protect themselves from snake bites (1). During the 9th century in China, *The Correct Treatment of Small Pox* was written by a Buddhist nun. The manuscript recommended a mixture of ground dried smallpox scabs and herb to be blown into the nostrils of children. Even with such a long history, immunization was not widely used until Edward Jenner deliberately injected cowpox virus into humans to protect them from ravages of smallpox. Since that time, wide use of vaccines against pathogenic microorganisms has become the most important advance in the history of medicine. Vaccines have not only provided protection from smallpox, but also from poliomyelitis, measles, mumps, rubella, yellow fever, pertussis (whooping cough), hepatitis A, hepatitis B, and varicella, as well as others. These vaccines have dramatically reduced morbidity from infectious agents and have directly protected more human lives than all other avenues of modern medicine combined. Yet, as increased standards for effectiveness and safety and the increased costs of developing and manufacturing vaccines become more restrictive, the development of new vaccines has slowed. Furthermore, as new pathogens continue to emerge, it is important that novel methods for vaccine production be developed and tested to meet more demanding requirements of the 21st century.

B. Vaccine Immunology

Traditional vaccines have relied on either live-replicating or nonliving preparations of microorganisms. The injected material functions as a vaccine by generating immunity against the inoculum, and the resulting immune responses function to prevent disease. This type of induced immunity is referred to as protective immunity and results from the vaccine activating specific B and T lymphocytes, which composes the lymphocyte subsets of the white blood cells of the immune system (2).

As the major components of humoral immune response, B cells are lymphocytes that develop in the fetal liver and subsequently mature in the bone marrow. Mature B cells carry surface immunoglobulins that act as their antigen-specific receptors. They move from the bone marrow through the circulation to secondary lymphoid tissues, the lymph nodes, and the spleen, where they respond to antigenic stimuli by dividing and differentiating into plasma cells under direction of cytokines produced by T cells. When they are activated, B lymphocytes become terminally differentiated to become plasma cells, which are entirely devoted to the production of secreted antibody. Antibodies are large water-soluble serum proteins, which are induced following contact with antigen. Antibodies bind to specific antigen, which drive their expansion and either directly neutralize or inactivate them. Antibodies can also direct other cells of the immune system, such as macrophages and phagocytes, to dispose of the antigen. Furthermore, they can direct complement, highly toxic, soluble immune mediators to bind to and destroy an invading pathogen.

T cells are lymphocytes that develop in the thymus. T cells acquire their antigen receptors in the thymus and differentiate into a number of subpopulations that have separate functions and that can be recognized by their different cell surface markers. T lymphocytes develop as 1 of 2 subsets of white blood cells termed T helper cells and T cytotoxic or T killer cells in the thymus. These cells become the basis of cell-mediated immune responses and function to eradicate pathogens in different ways. T helper cells help and direct B cells to produce antibody, which target mostly extracellular pathogens. T helper cells also cooperate with cytotoxic T lymphocytes in the destruction of virally infected cells. Activated T helper cells secrete small protein messengers termed cytokines or lymphokines, which activate and expand either or both humoral and cellular immune responses. However, activated T cytotoxic cells seek out and destroy cells that have been infected with pathogens. These T killer cells bind and destroy allogeneic and virally infected cells, which display recognizable antigen-MHC class I molecules. T killer cells induce these pathogen-infected cells to die either through the release of toxic proteins such as granzyme B or through initiating apoptosis or programmed cell death in the target cells. CD8 + cells can also mobilize the immune response at the point of antigen contact through the release of beta chemokines. In addition to defense against pathogens, cytotoxic T lymphocytes (CTL) are particularly important in eradicating misbehaving host cells such as cancer cells. Humoral or cellular immunity can act independently or in concert to destroy the pathogenic organism within a vaccinated host.

C. Traditional Vaccines

In the case of live vaccines, the infectious material has been weakened or attenuated so that it no longer induces disease. In the case of the nonlive preparations, the vaccine material has been manufactured to contain killed organisms or pieces of killed organisms that cannot grow when inoculated into a host. In some instances, specific components can be purified away from other portions of the microorganism or artificially manufactured in the laboratory to function as a subunit vaccine. Both categories of vaccines are presently used throughout the world to protect individuals against specific pathogens. Each has its own general characteristics for generating immunity and exhibits properties that can be beneficial or deleterious to an individual.

Live attenuated vaccines, such as the polio and smallpox vaccines, stimulate protective immunity as they replicate in the body of an immunized host. These vaccines emulate the natural infection of pathogens and generate a broad spectrum of immune responses. Because they are a weakened form of the pathogen, no disease occurs. This category of vaccine induces broad protective immunity with induction of both antibodies and activated T cells. More specifically, because CTLs are only induced if an infectious agent or vaccine actually is produced within host cells, live attenuated vaccines are the most effective inducer of CTL. Attenuated vaccines have an additional benefit in that they provide lifelong immunity. In contrast, nonliving inactivated vaccines (including subunit preparations), such as the vaccine for hepatitis A virus, produce protective immunity that is limited to the generation of antibodies and helper T cells, but cannot induce killer T cells. Accordingly, the ability of these preparations to induce protection is limited to pathogens that can be destroyed by extracellular defenses. Unlike the live attenuated vaccines, the protective immunity induced by inactivated vaccines is normally short term and requires repeated booster injections to achieve lifetime immunity. Based on these immunological characteristics, live attenuated vaccines represent the vaccines of choice. Still, they are not without problems. For instance, there are many safety issues related to the use of live attenuated vaccines. The potential for the attenuated vaccine to mutate back to the original disease-causing organism through a process is called reversion. Attenuated vaccines may also cause disease when inoculated into persons with weak or compromised immune systems, such as cancer patients receiving chemotherapy or acquired immune deficiency syndrome (AIDS) patients, or in the elderly where the immune system deteriorates with age. Furthermore, live vaccines can infect individuals other than the inoculated individual and thus inadvertently expose a disease to a susceptible unknowing individual.

Even though inactivated vaccines are safer than their live attenuated counterparts, certain problems also exist with some inactivated vaccines. For instance, the whole organism used as the inactivated vaccine can be contaminated with components from cell culture that are not removed during the manufacturing process. This contaminated material may be important in autoimmune disease. Additional shortcomings with inactivated vaccines include contamination by components of the pathogen that are not important in the generation of protective immunity. These components may generate immune responses that are not relevant to protective immunity. Deleterious reactions, such as inflammation and allergic reactions, may also result from vaccination with the inactivated whole organism. These concerns regarding contaminants and the safety issues related to whole organism vaccines point to the use of purified subunit component vaccines.

In these subunit vaccines, only the components of the microorganism involved in conferring protective immunity are included, whereas other portions of the microorganism are removed in an extensive purification process. This increases the cost of manufacturing the vaccine to improve its safety. Subunit component vaccines have an increased specificity that can target the immune response in a very effective manner; again these vaccines elicit protective immunity by the generation of antibodies and limited T helper responses. However, if antibodies alone are insufficient to provide protective immunity against a particular pathogen, it becomes necessary to also involve the activated T lymphocyte component of the immune response. As a need for vaccines against new pathogens emerges, safe vaccines that elicit both antibodies and activated T cells will have an advantage, particularly when the requirements for protective immunity in the host are not yet unknown. It would be a distinct advantage for vaccine development to have a technology that could induce the broad

immunity normally associated with a live attenuated vaccine, while exhibiting the safety and the focus of the subunit preparations. In addition, any simplification in manufacturing and increase in stability is likely to positively impact vaccine development for the developing world.

II. DNA VACCINES

A. Concepts of DNA Vaccines

Recent work from a number of laboratories has demonstrated that the injection of a DNA plasmid containing foreign genes for proteins of a pathogen or cancer antigens directly into a host results in the subsequent expression of the foreign gene in that host and the presentation of the specific encoded proteins to the immune system (Fig. 1). DNA vaccine constructs are produced as small circular vehicles or plasmids. These plasmids are constructed with a promoter site that starts the transcription process, an antigenic DNA sequence, and a messenger RNA stop site containing the poly A tract necessary for conversion of the messenger RNA sequence into the antigen protein by the ribosomal protein manufacturing machinery (Fig. 2). The concept of genetic immunization provides that both DNA and RNA that encode specific proteins can be used to generate specific immune responses. Because DNA and RNA are both nucleic acids, the term nucleic acid vaccine has also been used to describe this process.

B. History of DNA Inoculation

The ability of genetic material to deliver genes for therapeutic purposes and its use in gene therapy has been appreciated for some time. Early experiments describing DNA inoculation into living cells were DNA transfer experiments performed by a number of investigators in the 1950s and 1960s (3–5). These reports describe the ability of DNA preparations isolated from tumors or viral infections to induce tumors or virus infection following injection into animals. Importantly, many such inoculated animals developed antibody responses to the proteins encoded within the injected DNA sequences. Over the next 20 years, a number of scattered reports focusing on gene function or gene therapy techniques provided evidence that injection of viral DNA or plasmids containing foreign gene resulted in antibody production and was related to the DNA inoculations. In 1985, Dobensky reported that plasmids containing insulin DNA sequences could produce insulin following inoculation into a living animal for some period of time. Longer-term expression of foreign genes was described in 1990 by Wolff and colleagues following plasmid inoculation in vivo (6). These 2 separate observations demonstrated that DNA in the absence of vectors could deliver proteins that might have biological relevance. Although both studies focused on the use of this technology for gene replacement strategies, other studies were already underway in several laboratories using this same technology for vaccine applications.

In 1992, Johnston and colleagues at the Southwest Foundation reported that injection of DNA encoding human growth hormone into mice resulted in transient hormone production followed by the development of antibodies in the inoculated animals specific for the human growth hormone gene (7). This important work used a ''genetic gun'' or gene gun to shoot gold particles covered with DNA through the skin layers of mice. Although these investigators were actually studying the use of this technology for a gene therapy replacement strategy, they described this development of antibody responses due to this unusual immunization procedure as genetic immunization. Simultaneously with the publication by Johnston and colleagues, a vaccine meeting held at the Cold Spring Harbor Laboratory, Long Island, New York, in September 1992, described the use of DNA immunization to generate humoral and cellular immune responses against a human pathogen, as well as protection from both tumors and viral challenges in animal systems.

Investigators from Merck and Vical reported on the development of immune responses to intramuscular injected plasmid encoding pathogen proteins. They observed that both antibody responses as well as CTL responses were induced to influenza viral gene products by this immunization technique. Furthermore, vaccinated mice were able to resist lethal viral challenge. Harriet Robinson and her colleagues at the University of Massachusetts reported on the use of the gene gun to deliver influenza virus genes in DNA plasmids, indicating that both antibody and T lymphocyte responses were produced in vaccinated mice and in chickens. In challenge studies, these responses were protective. The use of the gene gun allowed investigators to deliver very low nanogram amounts of DNA at the site of injection and still observe immune responses. David Weiner and his colleagues at the University of Pennsylvania reported the direct injection of DNA encoding the genes for the human immunodeficiency virus (HIV). Again both antibodies and T lymphocyte responses specific for the viral gene products were observed in experimental animals. As HIV does not infect mice, an in vivo mouse model was used where tumor cells, which are normally lethal to the mice, were constructed to express HIV proteins. Animals who were vaccinated with the HIV DNA vaccine were demonstrated to be immune to these HIV antigen-expressing tumor cells. Although the audience was skeptical of the ability of nonliving genetic material to produce useful immune responses, the large amount of data presented by each of these groups representing several years of successful work in diverse systems could no longer be ignored by the scientific and vaccine community. DNA vaccines were officially born.

Following these initial reports, DNA vaccination and the generation of antibody and T lymphocyte responses, as well as protective responses in a variety of animal models, have been reported in the scientific literature for many human pathogens such as hepatitis B virus, rabies virus, herpes simplex virus, hepatitis C virus, human T cell leukemia virus, human papilloma virus, and tuberculosis (8–10).

C. Potential Advantages of DNA Vaccines

As summarized in Table 1, nucleic acid immunization may afford several potential advantages over traditional vaccina-

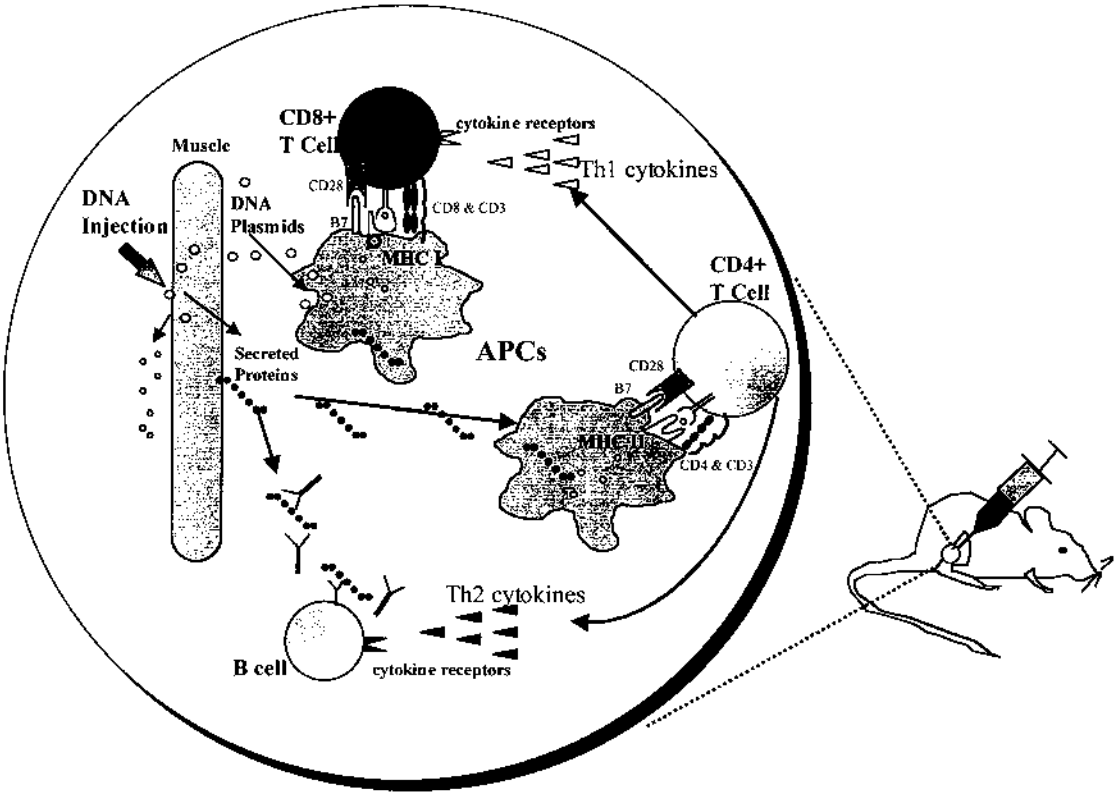

Figure 1 Induction of antigen-specific humoral and cellular immune responses following DNA immunization.

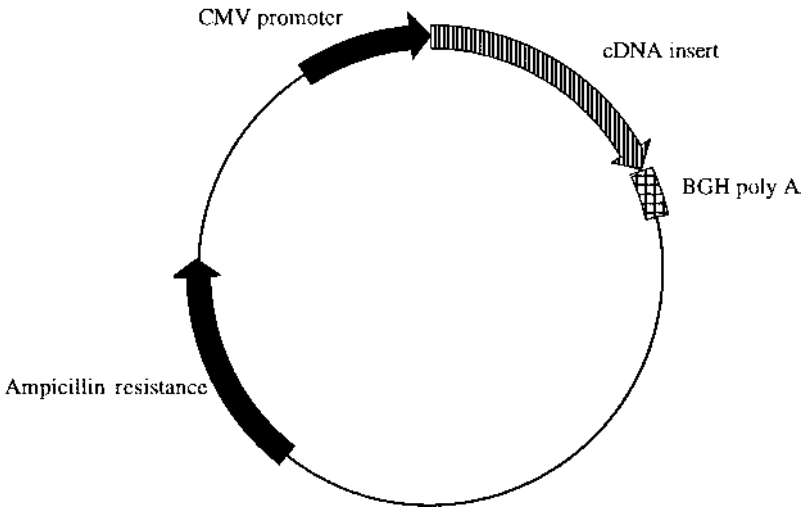

Figure 2 A diagram of a DNA vaccine construct consisting of a mammalian expression vector. The plasmids are constructed with a promoter, an antigenic DNA sequence, and a messenger RNA stop site containing the poly A tract.

Table 1 Summary of Immune Modulation by Molecular Adjuvants

	Molecular adjuvant	Immune modulation		
		Ab	Th	CTL
Proinflammatory cytokines	TNF-a	+++	++++	++++
	TNF-b	+	+	+
	IL-1a	+++	++	+/−
Th1 cytokines	IL-2	+++	++++	+
	IL-12	−	++++	++++
	IL-15	++	++	++++
	IL-18	+++	++++	++
Th2 cytokines	IL-4	++++	+	+/−
	IL-5	++++	++	+/−
	IL-10	++++	+++	+/−
Hematopoeitic cytokines	G-CSF	+	++	+
	GM-CSF	++++	++++	+
	M-CSF	++	+	++++
Chemokines	IL-8	+++	++++	+/−
	MIP-1a	++++	++	+/−
	RANTES	+	++++	++++
	MCP-1	+/−	+	++++
Costimulatory molecules	CD80	+/−	+	+/−
	CD86	+/−	++++	++++
	CD40	+/−	++++	++++
	CD40L	+/−	+	+

tion strategies, such as whole killed or live attenuated virus and recombinant protein-based vaccines, without specific shortcomings and inherent risks associated these vaccination methods. Like inactivated or subunit vaccines, DNA vaccines appear conceptually safe because they are nonreplicating and nonlive. In contrast to inactivated or subunit vaccine, DNA vaccine cassettes produce immunological responses that are more similar to live vaccine preparations. By directly introducing DNA into the host cell, the host cell is essentially directed to produce the antigenic protein, mimicking viral replication or tumor cell marker presentation in the host. Unlike a live attenuated vaccine, conceptually there is little risk from reversion to a disease-causing pathogen from the injected DNA, and there is no risk for secondary infection as the material injected is not living and not infectious. Furthermore, multicomponent DNA vaccines can be engineered to include specific immunogens, which could optimize and amplify desirable immunological responses. Perhaps this ability to target multiple antigenic components may be a particularly important characteristic of DNA vaccines because multicomponent DNA vaccines can be engineered to include specific immunogens that could optimize and amplify desirable immunological responses.

D. Mechanism of DNA Vaccines

The exact mechanism for DNA immunization has been a center of major debate (11–13), but is likely to be similar to traditional antigen presentation. In the body's immune system, cells need to process and present antigenic peptides to lymphocytes in order to stimulate antigen-specific immune response. Thus, antigen must be processed and presented to T lymphocytes by antigen-presenting cells (APCs) (14). Antigen presentation and recognition is a complex biological process that involves many interactions between antigen-presenting cells and T cells (Fig. 3). There are 4 primary components that are critical in the professional APC's ability to present the antigen to T cells and activate them for appropriate immune responses. These components are major his to compatibility complex [MHC]–antigen complexes, costimulatory molecules (primarily CD80 and CD86), intracellular adhesion molecules, and soluble cytokines. Naive T cells circulate through the body across lymph nodes and secondary lymphoid organs such as the spleen. Their migration is mediated among other factors by intercellular adhesion molecules and cytokines. As the T cells travel, they bind to and dissociate from various APCs. This action is mediated through adhesion molecules. When a naive T cell binds to an APC-expressing relevant MHC/peptide complex, the T cell expresses high levels of high-affinity IL-2 receptor. Only when this T cell receives a costimulatory signal through CD80/CD86–CD28 interaction does the T cell make soluble IL-2, which then binds to the receptors and drives the now-armed effector T cell to activate and proliferate.

Antigen is expressed at significant levels in muscle, following intramuscular inoculation of plasmid DNA (6). Using reporter gene injections in mice, various investigators have reported the detection of gene expression after intramuscular (IM) injection of DNA expression cassettes (6). Protein expression was detected in the quadriceps muscle of mice after injecting plasmid vectors encoding chloramphenicol acetyltransferase, luciferase, and β-galactosidase reporter genes into the muscle.

Muscle cells have several structural and functional features that seem to make them well suited for DNA uptake in vivo (15). Muscle consists of multinucleated contractile muscle fibers with cylindrical shape and tapered ends. These muscle fibers have mycogenic stem cells attached to them. When the muscle fibers are damaged or stressed, the stem cells are activated. The resulting myoblasts proliferate and eventually fuse to form new muscle fibers. It is believed that this continual activation and proliferation of the myoblasts allow a more opportunistic uptake of injected DNA. Because it has been shown that the uptake of the injected DNA and the subsequent production of protein occurs in muscle cells, they have been proposed as a potential site of antigen processing and presentation. However, the myocytes, which make up the muscle tissues, do not express CD80 or CD86 costimulatory molecules needed for efficient presentation, although a new study has identified an additional costimulatory molecule distinct from CD80 or CD86, which can be expressed in muscle cells (16). The question of ability of muscle cells to provide costimulatory signals drives the current debate in the literature about the mechanism of antigen presentation following intramuscular DNA immunization.

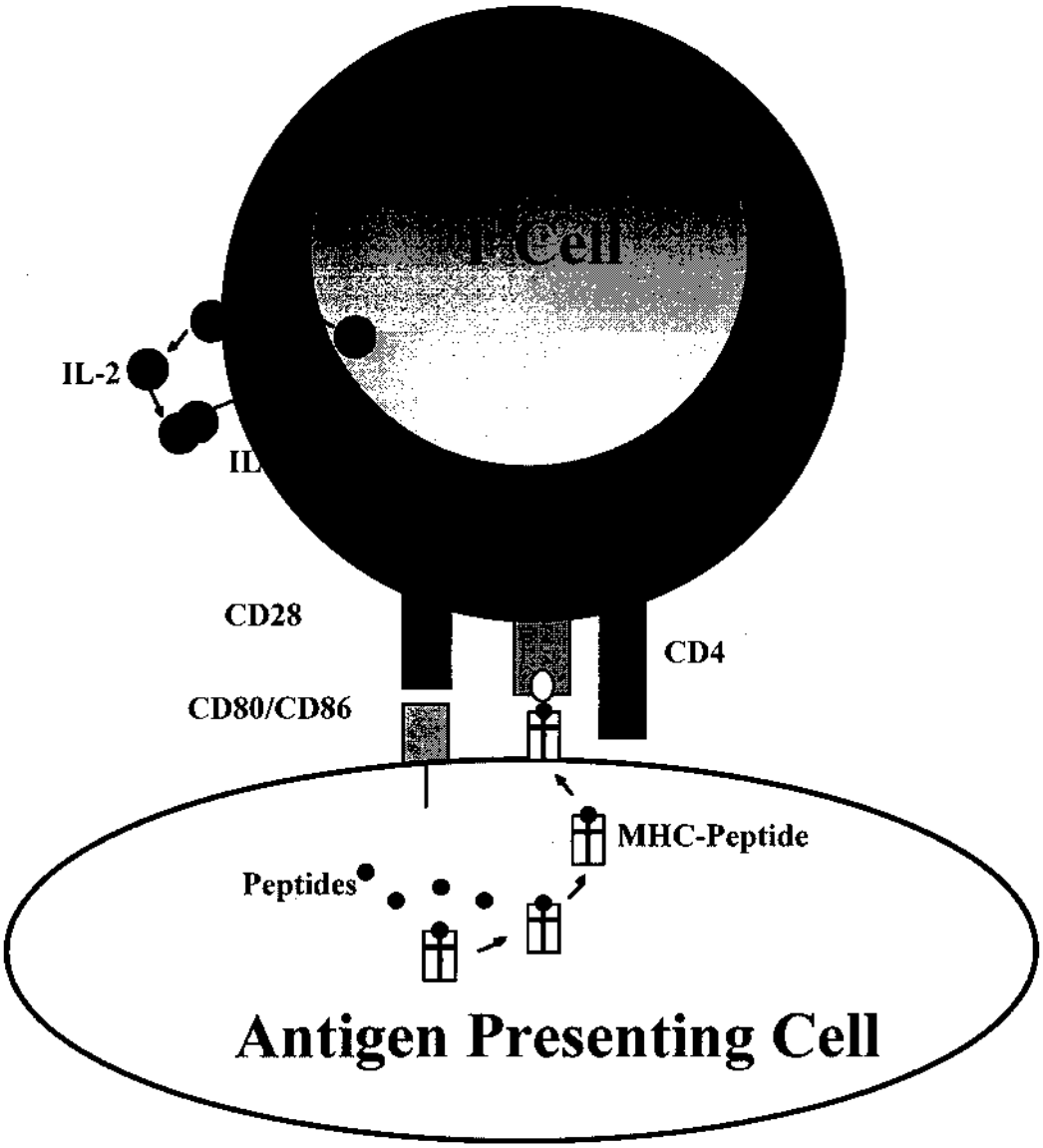

Figure 3 Effective T cell activation by APC. The interaction between antigen-MHC and T cell receptor leads to the expression of IL-2 receptor. This T cell proliferates when the second signal is provided from APC's costimulatory proteins. CD28-CD80/CD86 ligation initiates the production of IL-2 production and leads to the proliferation of activated T cell.

One potential mechanism is that the antigens produced in muscle are secreted from transfected muscle cells or released due to cell apoptosis (11,12). Such exogenous antigen could then be taken up by professional APCs in the draining lymph nodes, where the antigen is processed via the MHC class I pathway of these cells. Then, these APCs are hypothesized to present the processed peptides to T cells. Recently, there have been reports that indicate that immune system has an inherent mechanism by which exogenous antigens access MHC class I molecules. One recent report identified dendritic cells as the potent mediator of such presentation antigen derived from phagocytosed apoptotic cells (17). Immature dendritic cells engulf apoptotic cells and cross-present antigen from these sources to induce class I-restricted CTLs.

Another possible mechanism is the direct transfection of professional APCs by the injected DNA. Such a mechanism may be more probable in intradermal delivery of DNA because skin is rich in professional antigen-presenting cells, especially the dendritic cells. Condon et al. reported that through DNA immunization into skin, they were able to show expres-

sion of proteins encoded by DNA plasmids (18). However, such a mechanism is less likely within the muscle tissue, where there is significantly less presence of APCs. More recently, studies have reported that direct transfection of dendritic cells can occur following intramuscular inoculation DNA vaccine constructs, albeit at a lower level (19). Another study indicates that macrophages and dendritic cells may be a target cell for DNA transfected in vivo and that such a target might be important in driving immune responses in vivo (20). A clear understanding of the role of antigen-presenting cells in DNA vaccination could have important implications for this technology.

III. DNA VACCINES FOR INFECTIOUS DISEASES AND CANCER

A. DNA Vaccines Against HIV-1

The human immunodeficiency virus-1 (HIV-1) is a retrovirus, which preferentially infects and kills CD4+ T cells and macrophages, ultimately resulting in immune system failure and multipathogen infections. Recent breakthroughs in combination therapy using 3 or more different antiretroviral agents have generated optimism regarding the ability to control viral replication in vivo (21). However, this therapeutic regimen is costly, and it is too early to tell whether this approach can eradicate established infection (21,22). The costs and the stringent administration regimen requirements of these pharmaceutical agents make it clear that these drugs will only be effectively used in a limited part of the world population. Therefore, to address the worldwide problem of HIV-1 infection, there remains a need for a prophylactic vaccination strategy designed to control the epidemic through mass immunization campaigns (23).

One of the major obstacles in the development of a vaccine against HIV-1 is uncertainty regarding the exact immune correlates of protection (24). In studies of long-term nonprogressor groups of HIV-infected individuals, evidence supports the notion that correlates of protection against HIV-1 could be provided by humoral, cellular, or even both arms of the immune response (25,26). High levels of type-specific neutralizing antibody have been observed in protected primates in some homologous challenge models (27–31). Neutralizing antibodies are susceptible to viral deception through antigenic diversity of HIV-1 envelope, and the ability of neutralizing antibody to prevent viral pathogenesis is still under considerable investigation (32–35).

One of the hallmarks of HIV-1 disease progression is the loss of cellular immune function, and the presence of strong cellular responses in some instances can correlate with control of viral replication (9,36). In cases of acute HIV-1 infection studied by several investigators, viral clearance was associated with specific CTL activity in each case (37,38). In addition, a subset (7 of 20) of occupationally exposed health care workers who were not infected possessed transient HIV-1-specific CTL response (39). HIV-1-specific CTLs were also found in a number of chronically exposed sex workers in Gambia who continue to resist infection with HIV-1 (40). In spite of these studies supporting the role of neutralizing antibodies and CTLs in conferring immunity to infection, some vaccinated primates exhibiting both neutralizing antibody and CTL responses were not protected from subsequent viral challenge in the pathogenic SIV model (41). Recently, the important role of CD4 helper responses in the anti-HIV immune response has been highlighted (42). Such responses likely have importance for both humoral and CD8+ effector responses.

The advantages of nucleic acid immunization listed above make it well suited as a potentially useful vaccination strategy against HIV-1. Within the HIV genome, there are several potential immunological targets for DNA vaccination (Fig. 4). The HIV-1 genome is organized into 3 major structural and enzymatic genes, 2 regulatory genes, and 4 accessory genes (43). The first major gene target is *env*, which codes for the outer viral envelope proteins. HIV enters the CD4+ cells via envelope–CD4 receptor complex. Following entry of HIV viral core, synthesis of a double-stranded DNA version of the HIV genome (called DNA provirus) begins by the viral DNA polymerase, reverse transcriptase (RT). The DNA provirus is then translocated to the nucleus as part of the protein–DNA preintegration complex and is integrated into the host cell genome with the help of the viral integrase (Int) enzyme. The provirus then replicates with the host DNA each time the cell divides. The gene *gag* codes for the core protein, and *pol* codes for the enzymatic proteins RT, Int, and protease (Pro). In general, these enzymatic proteins remain somewhat conserved and preserve their catalytic functions. Accordingly, these proteins may be less divergent immune targets than envelope proteins for CTL-mediated responses (44).

The regulatory genes *tat* and *rev* affect HIV-1 gene expression. Viral transcription is increased several hundred-fold by tat transactivation (45), making it an obvious target for therapeutic intervention. The rev protein increases the release of unspliced structural RNAs from the nucleus by displacing host splicing factors that otherwise prevent RNA transport from the nucleus to the cytoplasm (46). In addition, rev is a critical component in the production of the structural proteins of HIV-1. A report of a DNA vaccine study in humans shows the immunogenicity of the *rev* gene product, again supporting its importance as a vaccine target (47). In addition, a *Tat* DNA vaccine has been reported to influence SHIV viral challenge in a macaque model system, while human immune response against HIV infection includes *Vpr* as a target (48,49). However, studies with *Vpr* as a plasmid vaccine component report that its presence can negatively influence the resulting host immune response (50,51). Therefore, this target requires more study before being included as part of a plasmid vaccine cocktail. Nonetheless, further work on these targets could be important as they and Nef are the earliest HIV antigens expressed. Targeting these antigens may give the immune system early warning of HIV challenge.

In addition to the regulatory genes, HIV-1 carries an additional set of accessory genes, *vif* (virion infectivity factor), *vpr* (viral protein r), *vpu* (viral protein u), and *nef* (negative factor), which are potential targets for DNA immunization.

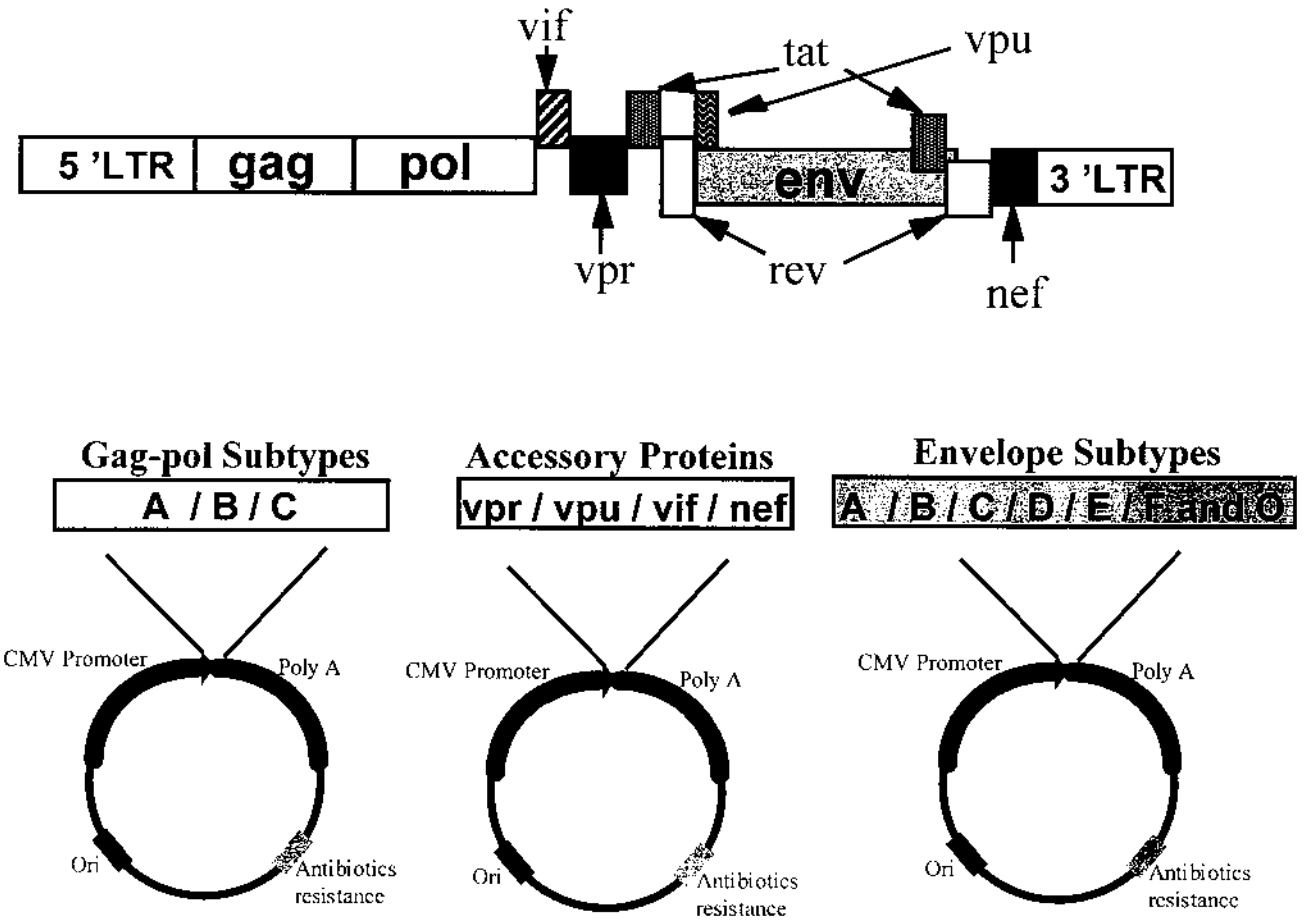

Figure 4 Potential immunological targets for DNA vaccination against HIV-1. These targets include *env*, *gag*, and *pol* genes, as well as the 4 accessory genes.

These accessory genes can be deleted from the viral genome without eliminating replication in vitro, suggesting that these gene products play a secondary rather than primary role in viral infection. The vif protein is located in the plasma membrane and may be important for production of infectious virions (52). In contrast to vif, vpu seems to facilitate the degradation of intracellular CD4 molecules (52). The vpr protein is found in the viral particle in high amounts and appears to have several biological activities, including the ability to increase viral transcription and to reactivate virus from cellular latency and arrest host cell division (53–55). Nef has never been critical for viral infection of cell lines in vitro (56), although experiments performed with the related simian immunodeficiency virus (SIV) found that rhesus macaques infected with virus having a deletion in *nef* had dramatically lower levels of viral replication (57). In addition, recent evidence suggests that *Nef* plays a crucial role in mediating bystander apoptosis through the upregulation of FasL expression on infected T cells (58). Accordingly, *Nef* expression in T cells also suppresses cell death by blocking ASK1-mediated cell death and by inducing signals that phosphorylate BAD (59,60). Developing DNA vaccine constructs directed against these accessory genes could provide additional arsenal in our battle against HIV-1.

DNA expression cassettes encoding for HIV-1 envelopes (strains HXB2, -MN, and -Z6) were among the first to be analyzed for immunogenicity (Fig. 4) (61). Initial studies demonstrated that mice immunized with envelope constructs produced antibodies specific to recombinant gp160, gp120, and gp41 proteins. The antisera neutralized HIV-1 isolates in vitro at a low level (62). Neutralization of homologous isolates has also been reported after immunization with constructs based on the HIV-1 NL4-3 isolate in the presence of relatively low antienvelope IgG titers (63). Moreover, the pM160-MN construct not only demonstrated neutralization of homologous isolates but also showed lower, yet measurable neutralization of the heterologous HIV-1 Z6 isolate (62). In addition to the humoral responses, cellular immune responses were observed from envelope inoculated mice. Induction of T helper cell

proliferative response against recombinant gp120 protein was observed (61). In addition, cytotoxic T lymphocyte responses have been observed against both targets infected with recombinant vaccinia-expressing envelope protein and targets prepared with envelope peptides (61,63,64).

In contrast to the high level of sequence divergence observed in the envelope glycoproteins of HIV-1, their *gag* and *pol* gene sequences appear to be less variable immunological targets. Thus, combining env constructs with gag/pol constructs could result in a more potent vaccination program. Expression cassettes encoding for both gag and pol elicited antigen-specific antibody, Th, and CTL responses (65). In addition to the DNA immunogen cassettes encoding env and gag/pol proteins, the DNA expression cassettes targeting nef and vif accessory proteins have been developed, and they have been shown to induce both antigen-specific humoral and cellular responses in mice (66,67).

For HIV plasmid vaccines, the issue of *rev* independence has appeared as a recent important issue. The structural genes of HIV are made as long unspliced transcripts that contain overlapping reading frames with the small regulatory genes of HIV *tat* and *rev*. These transcripts are retained in the nucleus and rapidly spliced to encode the small regulatory in the absence of *rev*, thus preventing the transport of full-length message to the ER where structural gene translation can occur. The messages are inhibited from transport to the ER by both known and cryptic *rev*-dependent sequences. In view of this, recent studies suggest that optimization of codons to the usage of highly expressed human genes, as well as deletion of residual inhibitory sequences, significantly enhanced Gag expression leading to potent augmentation of immune responses (68,69). In addition, a recent study suggested that alteration of AT-rich regions without changing the amino acid sequences can significantly enhance Gag expression through *rev*-independent mechanisms. These modifications led to enhanced Gag-specific immune responses, indicating that codon optimization may provide new avenues for enhancing vaccine potencies (70).

B. DNA Vaccines Against Other Viruses

In addition to HIV-1, there is a growing list of DNA vaccines targeted against other viral pathogens. One of its central attractions is its flexibility in modulating immune responses. As a consequence, several vaccine cocktails have been created that specifically target highly immunogenic antigens, which result in potent antiviral immune responses.

The West Nile virus (WNV) is a vectorborne pathogen that induces brain inflammation and death in endemic regions. Recently, confirmed cases of infection and death have been reported in the Mid-Atlantic region of the United States. Because there is no specific therapy for the WNV infection at this moment, there is an increasing demand for the development of vaccine strategies to prevent disease from this virus. Currently, no human or veterinary vaccine is available to prevent WNV infection, and mosquito control is the only practical strategy to combat the spread of disease. Several vaccine companies, including Acambis, Inc., and Baxter/immuno, have research and development programs on human vaccines (71). One major veterinary vaccine manufacturer (Ft. Dodge) is also developing formalin-inactivated and naked DNA vaccines.

A group from the Centers for Disease Control and Prevention has reported on the induction of protective immunity using a DNA vaccine that expressed the WNV prM and E proteins (72). In addition, as an extension of the multicomponent DNA vaccines strategy, a DNA vaccine expressing the WNV capsid (Cp) protein has been shown to induce immune responses in DNA vaccine-immunized mice (73). Collectively, these results support the potential utility of DNA vaccines as a tool for developing immunization strategies for WNV and other emerging pathogens.

Some of the earliest DNA vaccines were against influenza. In fact, the initial report by Ulmer et al. targeted the nucleoprotein as an antigen and effectively generated CTLs to achieve protection against subsequent challenge with a heterologous strain of influenza A virus (74). Protection was also achieved through immunization against the hemagglutinin antigen (75). Interestingly, cross-protection of variant viral strains was also achieved in both mice and preclinical studies (76,77).

Other exciting prospects for DNA vaccines against viruses include complete protection against measles in a macaque model. Surprisingly, protection correlated with the generation of neutralizing antibodies rather than cytotoxic T cell induction (78).

Finally with the advent of recent bioterrorism scares, much interest has focused on the development of rapid mass stocks of vaccines to immunize the general public (79). In view of this, DNA vaccines' simplicity and ease of mass production effectively overcomes such obstacles. In addition, plasmid immunization against the protective antigen and lethal factor of anthrax effectively induced complete protection in preliminary mice experiments (80,81). Similar results were also achieved against the Ebola virus in both mice and primate experiments (82). These preliminary results highlight the potential efficacy of DNA vaccines for generating mass immunizations against potential bioterroristic weapons.

C. DNA Vaccines for Cancer

Although advances in science have led to countless theories and methods designed to combat human carcinoma, the battle is far from over. Surgical excision of tumors, drug therapies, and chemotherapy have been effective in certain cases but in other situations, particularly when the tumor has begun to metastasize, effective treatment is far more difficult and far less potent. Thus, researchers are continually investigating novel and more effective treatment strategies for various forms of cancer. Research, in recent years, has turned toward the use of vaccines to treat cancer. To this end, several proteins produced by tumor cells became a target for vaccine development. These tumor-associated antigens are predominantly expressed in a tissue-specific manner and are expressed at greatly increased levels in affected cells. Besides being impor-

tant diagnostic aids, these antigens represent appropriate targets for the development of cancer vaccines (83).

Tumor-associated antigens (TAAs) are proteins produced by tumor cells that can be presented on the cell surface in the context of major histocompatibility complexes (84). Recently, these antigens have been the focus of study as a viable option for immunotherapy of various types of cancer. In this chapter, we examine the progress in the investigation of the immunological effects of 3 such TAAs, carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), and human papillomavirus (HPV) type 16 E7 antigen.

D. DNA Vaccines Against Colon Cancer

Human CEA is a 180-kDa glycoprotein expressed in elevated levels in 90% of gastrointestinal malignancies, including colon, rectal, stomach, and pancreatic tumors, 70% of lung cancers, and 50% of breast cancers (84,85). CEA is also found in human fetal digestive organ tissue, hence the name carcinoembryonic antigen (86). It has been discovered that CEA is expressed in normal adult colon epithelium as well, albeit at far lower levels (87,88). Sequencing of CEA shows that it is associated with the human immunoglobulin gene superfamily and that it may be involved in the metastasizing of tumor cells (86).

The immune response to nucleic acid vaccination using a CEA DNA construct was characterized in a murine model. The CEA insert was cloned into a vector containing the cytomegalovirus (CMV) early promoter/enhancer and injected intramuscularly. CEA-specific humoral and cellular responses were detected in the immunized mice. These responses were comparable to the immune response generated by rV-CEA (87). The CEA DNA vaccine was also characterized in a canine model, where sera obtained from dogs injected intramuscularly with the construct demonstrated an increase in antibody levels (89). Cellular immune responses quantified using the lymphoblast transformation assay also revealed proliferation of CEA-specific lymphocytes. Therefore, a CEA nucleic acid vaccine was able to induce both arms of the immune responses (89). CEA DNA vaccines are currently being investigated in humans.

E. DNA Vaccines Against Prostate Cancer

Prostate cancer is the most common form of cancer and the second most common cause of cancer-related death in American men (90). The appearance of prostate cancer is much more common in men over the age of 50 (91). Three of the most widely used treatments are surgical excision of the prostate and seminal vesicles, external bean irradiation, and androgen deprivation. However, conventional therapies lose their efficacy once the tumor has metastasized, which is the case in more than half of initial diagnoses (92,93).

PSA is a serine protease and a human glandular kallikrein gene product of 240 amino acids, which is secreted by both normal and transformed epithelial cells of the prostate gland (94,95). Because cancer cells secrete much higher levels of the antigen, PSA level is a particularly reliable and effective diagnostic indicator of the presence of prostate cancer (96). PSA is also found in normal prostate epithelial tissue and its expression is highly specific (97).

The immune responses induced by a DNA vaccine encoding for human PSA has been investigated in a murine model (97). The vaccine construct was constructed by cloning a gene for PSA into expression vectors under control of a CMV promoter. Following the injection of the PSA DNA construct (pCPSA), various assays were performed to measure both the humoral and cellular immune responses of the mice. PSA-specific immune responses induced in vivo by immunization were characterized by enzyme-linked immunosorbent assay (ELISA), T helper proliferation CTL, and flow cytometry assays. Strong and persistent antibody responses were observed against PSA for at least 180 days following immunization. In addition, a significant T helper cell proliferation was observed against PSA protein. Immunization with pCPSA also induced MHC class I CD8+ T cell-restricted CTL response against tumor cell targets expressing PSA. The induction of PSA-specific humoral and cellular immune responses following injection with pCPSA was also observed in rhesus macaques (98).

F. DNA Vaccines Against Cervical Cancer

HPV 16-associated proteins, including E6 and E7, are some of the most common proteins in cervical cancers and are ubiquitiously expressed within these cells (99,100). However, DNA-based vaccine targeting these proteins seem to elicit minimal immune responses, and may necessitate potent adjuvants to provide efficacious tumor protection. Accordingly, recent studies suggest numerous targeting mechanisms (i.e., adjuvants) to enhance immunogenicity of these antigens.

Heat shock proteins (HSPs) have been found to induce tumor immunogenicity and their levels of expression are enhanced with highly immunogenic necrotic bodies (101–103). HSP–peptide complexes are also highly immunogenic and function effectively as adjuvants and cross-priming proteins (104–107). It has also been reported that the enhanced CTL responses are CD4+ independent and are limited only to antigenic peptides associated with HSPs (108–110). Importantly, evidence suggests that the HSPs may specifically target immature dendritic cells for the induction of proinflammatory reponses, further validating their potential role as vaccine adjuvants (111). In view of this, the HSP70 of mycobacterium tuberculosis was fused to the HPV-16 E7 antigen to construct a chimeric DNA vaccine. The E7-HSP70 DNA vaccine induced significantly enhanced levels of cellular responses, including a ratio of 435:14 (E7-HSP70 to E7) of E7-specific IFN-γ spot-forming CD8+ T cells via ELISPOT assays. In addition, data indicate the eradication of preexisting tumors and the resulting response was via CD4+-independent mechanisms (111). The exact mechanism of HSP-mediated peptide processing is still yet unclear, although enhanced proteasomal processing may be involved in this cross-priming maneuver (111). In a similar fashion, another member of the HSP family that has aug-

mented the potency of DNA vaccines is calreticulin (112). The idea of calreticulin as an immune modulator was based on previous findings that calreticulin in conjugation with tumor peptides stimulates potent peptide-specific CD8+ T cell responses (113). In addition, calreticulin and its fragment vasostatin operate as inhibitors of angiogensis (114,115). Accordingly, when calreticulin was fused to HPV-16 E7 antigen as a DNA vaccine, a potent antitumor effect was provoked. The resulting response was attributed to both the enhanced immunogenicity against E7 and the generation of antiangiogenesis (112).

A more recent report within the clinics also suggests that immunization through DNA can also therapeutically attenuate the growth of neoplastic cells in humans. These studies specifically encapsulated DNA plasmids encoding HLA-A2-restricted epitopes of the HPV E7 antigen within biodegradable polymer microparticles. Early work suggests no adverse side effects while enhancing immune responses when implementing this specific therapy (116). All together, these promising results emphasize the potential of DNA vaccines as therapies.

IV. INDUCTION OF IMMUNE RESPONSES IN PRIMATES

It would be desirable to evaluate in primates DNA vaccine constructs that induced high levels of immune responses in mice. Nonhuman primates represent the most relevant animal challenge model for HIV vaccine studies. Specifically, there are currently 3 different primate models for HIV vaccine studies. They include the HIV challenge model in chimpanzees and the SIV and chimeric SIV/HIV-1 (SHIV-1) challenge models in macaques. Chimpanzees can be infected by HIV isolates from humans, although they do not readily develop AIDS-like disease. However, the SIV challenge model uses the macaque SIV, which replicates to high levels and causes an AIDS-like disease in both cynomolgus and rhesus macaques. The chimeric SHIV viruses were constructed by replacing SIV envelope genes with specific HIV-1 envelope genes (117). The SHIV viruses replicate in macaques similarly to SIV and represent an infectious challenge model for HIV-1 envelope-based vaccines. Importantly, certain SHIV strains such as SHIV 89.6P are pathogenic.

DNA vaccination has been shown to induce both strain-specific neutralizing antibodies as well as antigen-specific T cell responses in both macaques and chimpanzees. The ability of DNA vaccines to provide protective immunity from viral challenge in primates has had mixed results. In a chimpanzee HIV challenge model, 2 out of 2 chimpanzees inoculated with constructs encoding for HIV envelope and gag/pol proteins from strain MN were protected from an intravenous (IV) challenge with a high dose (250 chimpanzee ID_{50}) of a heterologous stock of HIV-1 SF2 (118). In an early macaque study, 4 cynomolgus monkeys immunized with 2 different HIV-1 envelope constructs (encoding subtypes B and D) resulted in an induction of antigen-specific humoral responses, including neutralizing antibodies and cellular responses such as proliferative and cytotoxic responses (119). However, only 1 of 4 cynomolgus macaques were protected from IV challenge with 50 $TCID_{50}$ of a SHIV-1 HXB2 chimeric virus stock. More recently, 2 out of 2 rhesus monkeys primed with large doses of HIV-1 gp120 DNA vaccine constructs and boosted with gp160 protein were protected from an IV challenge with 25 $TCID_{50}$ of SHIV-1 HXB2 (120). However, protein vaccines alone can protect in this model in a type-specific fashion and protection is based on the ability of protein to boost the type-specific neutralizing antibody response. More recently, however, priming with gp160 DNA and boosting with recombinant protein did not result in protection in the identical SHIV model (121). Thus, the effectiveness of this vaccine strategy is unclear in the macaque model. Early reports have suggested that complete protection against SHIV could be achieved though when coimmunized with an IL-2-F_c fusion cytokine (183). Monkeys that were immunized with the IL-2-F_c cytokine exhibited potent CTL induction and stable CD4+ T cell counts, while minimal to no viral loads were detected (183). In addition to these DNA and protein prime/boost studies, other prime/boost strategies using DNA and recombinant viruses (e.g., recombinant poxvirus and adenovirus) are being investigated. Cellular immune responses have been reported to enhance with such prime/boost strategies (122), their effects on viral protection have been generated through the boosting with both MVA and Ad5 models (201,205).

The protective effects of DNA vaccine constructs in the SIV challenge model have been significantly less encouraging. Seven rhesus macaques were immunized with DNA vaccines encoding both envelope (4 different plasmids) and gag (1 plasmid) genes of SIV and were challenged with pathogenic SIV_{mac251} after their sixth immunization. Although vaccines induced positive responses, none of the vaccinated animals were protected from infection or disease (123).

More recently, we immunized rhesus macaques with DNA vaccine constructs encoding for HIV env/rev and SIV gag/pol proteins (124). Vaccinated animals were challenged intravenously with SHIV IIIB. Half of the animals in the vaccine group exhibited protection from infection based on sensitive limiting dilution coculture, demonstrating a dramatic effect on viral replication of the vaccines tested. The protected animals were reboosted with SIV DNA vaccines and were rechallenged intravenously with pathogenic SIV_{mac239}. All vaccinated animals were negative for viral coculture and antigenemia and remained healthy.

Whether the protection from SIV challenge is entirely due to DNA vaccines alone or due to DNA and SHIV challenge should be further studied. It is important to consider the role of SHIV challenge as a boosting agent for gag-specific cellular responses in this study. Regardless, these results demonstrate that protection from pathogenic challenge can be achieved in the absence of viral replication that reaches a threshold level of replication for effective vaccination, and these findings could be important in assessing relevant multicomponent vaccination strategies for HIV.

V. SAFETY AND EFFICACY STUDIES IN HUMANS

The ultimate goal of vaccine development is demonstrated safety and efficacy in humans. The first DNA vaccine studies to enter the clinic were DNA vaccines encoding for HIV-1 MN envelope (125). Fifteen healthy HIV-1 seropositive volunteers with greater than 500 CD4+ lymphocytes/mL were enrolled in this study. Patients in the trial received 3 injections each separated by 10 weeks with escalating dosage (3 dosage groups of 5 subjects) of envelope vaccine. Preliminary results reveal no significant clinical or laboratory adverse effects measured in all 3 dosage groups (30, 100, and 300 μg). More important, the immunized individuals developed an increase in antibody responses to envelope proteins and peptides after receiving the 100-μg dose. Some increases in cellular responses, including the lymphoproliferative and CTL responses, as well as β-chemokine expression were also observed (125).

These preliminary results demonstrate that the injection of even relatively low doses of a single immunogen DNA vaccine is capable of augmenting both existing humoral and cellular immune responses in humans. In addition to the initial human trials, phase I trials evaluating a gag/pol construct as a therapeutic vaccine as well as a prophylactic DNA vaccine study for HIV, have been undertaken. In another clinical study, the healthy volunteers who were immunized with DNA vaccines encoding for malaria proteins developed CTL responses against the target cells prepared with malaria peptides (126). Taken together, these studies are dramatically expanding our knowledge of DNA vaccines for clinical use in humans.

VI. MOLECULAR ADJUVANTS AS AN IMMUNE MODULATION STRATEGY

The primary goal of the first-generation DNA immunization studies was to demonstrate and evaluate the DNA vaccines' ability to elicit humoral and cellular responses in vivo in a safe and well-tolerated manner. As we explore the next-generation of DNA vaccines, it would be desirable to refine current DNA vaccination strategies to elicit more clinically efficacious immune responses. In this regard, the next generation nucleic acid vaccines may require better control of the magnitude and direction (humoral or cellular) of the immune responses induced. Such modulation of immune responses can be accomplished by the use of genetic adjuvants. Genetic or molecular adjuvants are different than the traditional adjuvants in that they are comprised of gene expression constructs encoding for immunologically important molecules. These molecules include cytokines, chemokines, and costimulatory molecules (9). These molecular adjuvant constructs could be coadministered along with immunogen constructs to modulate the magnitude and direction (humoral or cellular) of the immune responses induced by the vaccine cassettes themselves (Fig. 5). Such use of molecular adjuvant constructs results in concurrent kinetics of in vivo expression for both the adjuvant and antigen proteins. Perhaps this ability to engineer targeted

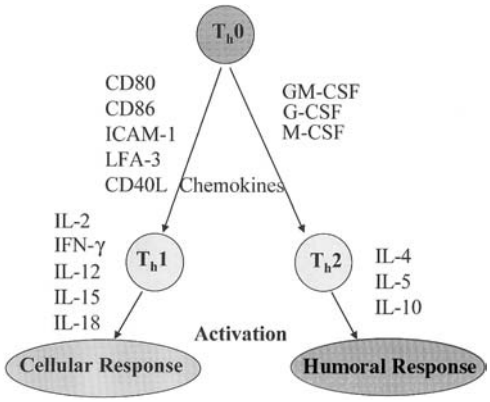

Figure 5 Cytokines, chemokines, and costimulatory molecules as immune regulators. Cytokines, chemokines, and costimulatory molecules play critical roles in the immune and inflammatory responses. Based on their specific function in the immune system, these cytokines could be further grouped as proinflammatory, Th1, and Th2 cytokines. Along with costimulatory molecules and chemokines, these cytokines also play important roles in the activation and proliferation of T and B cells.

immune responses may be a particularly important aspect of a multicomponent DNA vaccine strategy.

A. Cytokine Molecular Adjuvants

To focus the immune responses induced from DNA vaccines, we and others have investigated the codelivery of molecular adjuvants to modulate vaccine responses (65,127–135). We initially reported that coimmunization of GM-CSF cDNA with DNA vaccine constructs increases antigen-specific antibody and T helper cell proliferation responses, while coimmunization with IL-12 cDNA results in weaker antibody responses and enhanced T helper cell proliferation in mice (65,127). In addition, IL-12 coimmunization resulted in a significant enhancement of CTL responses. Importantly, we observed a significant enhancement of CTL response in vivo with the coadministration of murine IL-12 genes with 4 different HIV-1 DNA immunogens (*gag*/*pol*, *envelope*, *vif*, and *nef*), which were CD8+ T cell and MHC class I restricted. In contrast, almost no effect on CTL induction was observed with the genes for GM-CSF in these studies. Iwasaki et al. reported a similar finding using GM-CSF and IL-12 codelivery with DNA immunogen encoding for influenza NP.

More recently, we investigated the induction and regulation of immune responses from the codelivery of proinflammatory cytokines (IL-1α, TNF-α, and TNF-β), T_h1 cytokines (IL-2, IL-15, and IL-18), and T_h2 cytokines (IL-4, IL-5, and IL-10) (128). We observed that some Th1, as well as Th2, cytokine genes increased the antibody response, specifically coinjection with IL-2, IL-4, IL-5, IL-10, and IL-18, all resulted in increased levels of antibodies. We also found that coinjec-

tion with TNF-α, TNF-β, IL-2, IL-10, and IL-18 resulted in a dramatic enhancement of T helper proliferation response, while coinjection with IL-5 and IL-15 resulted in a more moderate increase in T helper proliferation. Furthermore, among all coinjection combinations, we found that only TNF-α and IL-15 coinjections resulted in a high level of CTL enhancement similar to that of IL-12 coinjection. Coinjection with TNF-β, IL-2, and IL-18 resulted in a more moderate increase in CTL response over those groups immunized with only DNA immunogen. As observed with IL-12 coinjection, the enhancement of CTL responses observed from the coinjections with TNF-α and IL-15 were restricted by MHC class I and CD8 + T cell dependent.

We also investigated whether the Th1- or Th2-type immune responses are more important for protection from HSV-2 infection (136). We codelivered DNA expression construct encoding for HSV-2 gD protein with the gene plasmids encoding for Th1-type (IL-2, IL-12, IL-15, IL-18) and Th2-type (IL-4, IL-10) cytokines in an effort to drive immunity induced by vaccination. We then analyzed the vaccine modulatory effects on resulting immune phenotype and on the mortality and morbidity of the immunized animals, following HSV lethal challenge (Fig. 6). We observed Th1 cytokine gene coadministration not only enhanced survival rate, but also reduced the frequency and severity of herpetic lesions following intravaginal HSV challenge. However, coinjection with Th2 cytokine genes increased the rate of mortality and morbidity of the challenged mice. Again, among the Th1-type cytokine genes tested, IL-12 was particularly a potent adjuvant for the gD DNA vaccination, resulting in increased survival and decreased animal morbidity.

B. Chemokine Molecular Adjuvants

Similar to cytokine gene codelivery, we found that the coimmunization with chemokine genes along with DNA immunogen constructs can modulate the direction and magnitude of induced immune responses (130). We observed that coimmunization with IL-8 and MIP-1α genes increased the antibody response in a similar manner to IL-4 or GM-CSF coimmunization. We also found that coinjection with IL-8 and RANTES resulted in a dramatic enhancement of T helper proliferation response. Among all coinjection combinations, we found that RANTES and MCP-1 coinjections resulted in a high level of CTL enhancement, almost as significant as IL-12, a potent CTL inducer for DNA vaccines. In addition, there is evidence that suggests some chemokine adjuvanted vaccines require a direct fusion of the immunogen with the chemokine. For instance, immunization against HIV gp120 with monocyte chemoattractant protein-3 (MCP-3/CCL7) or macrophage-derived chemokine (MDC/CCL22) requires the direct fusion of the 2 for effective immune response induction. Accordingly, coimmunization revealed minimal immune enhancement (137). Therefore, the use of these chemokine vaccines could be particularly important as HIV vaccine modulators of β-chemokines. In this regard, we observed that β-chemokines as vaccine adjuvants augmented β-chemokine production in a vaccine antigen-specific manner. This aspect could be especially important for a development of a vaccine that modulates the earliest aspects of the inflammatory response.

C. Costimulatory Molecule Molecular Adjuvants

Professional APCs initiate T cell activation through binding of antigenic peptide–MHC complexes to specific T cell receptor molecules. In addition, the APCs provide critical costimulatory signals to T cells, which are required for the clonal expansion and differentiation of T cells. Among different costimulatory molecules, B7 molecules (CD80 and CD86) have been

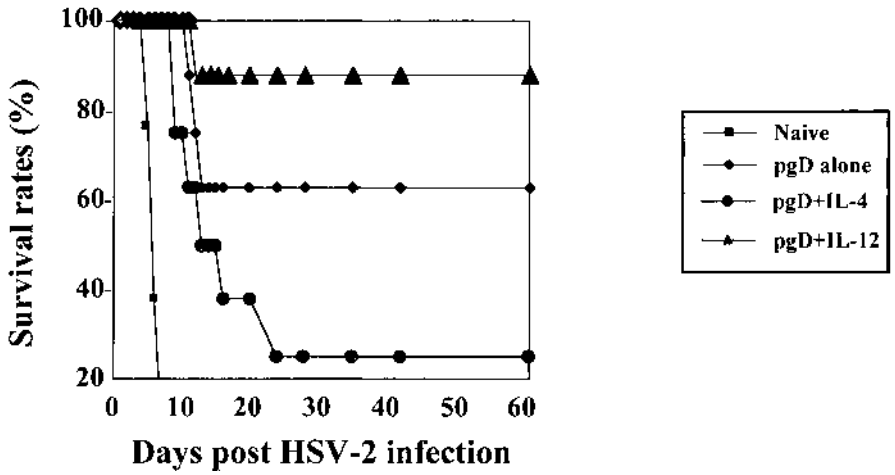

Figure 6 Protection from lethal HSV-2 challenge. Each group of mice ($n = 10$) was immunized with gD DNA vaccines (60 μg), and/or cytokine genes (40 μg) at 0 and 2 weeks. Three weeks after the second immunization, mice ($n = 8$) were challenged intravenously with 200 × LD_{50} of HSV-2 strain 186 (7×10^5 pfu).

observed to provide potent immune signals (138,139). They bind to their receptors (CD28/CTLA-4) present on T cells. The CD80 and CD86 molecules are surface glycoproteins and members of the immunoglobulin superfamily, which are expressed only on professional APCs (138–140). The blocking of this additional costimulatory signal leads to T cell anergy (141).

We reported that CD86 molecules play a prominent role in the antigen-specific induction of $CD8^+$ CTLs when delivered as vaccine adjuvants (131). Coadministration of CD86 cDNA along with DNA-encoding HIV-1 antigens intramuscularly dramatically increased antigen-specific T cell responses without a significant change to the level of the humoral response. This enhancement of CTL response was both MHC class I restricted and CD8+ T cell dependent. Similar results have been obtained by other investigators who also found that CD86, not CD80, coexpression results in the enhancement of T cell-mediated immune responses (133,134).

Accordingly, we speculated that engineering of nonprofessional APCs such as muscle cells to express CD86 costimulatory molecules could empower them to prime CTL precursors. However, the enhancement effect of CD86 codelivery could also have been mediated through the direct transfection of a small number of professional APCs residing within the muscle tissue. Subsequently, these cells could have greater expression of costimulatory molecules and could in theory become more potent. To investigate this issue, we constructed a set of bone marrow chimeric animals between normal mice and mice bearing a disrupted β_2-microglobulin (β_2m) gene (142). These bone marrow chimeras could respond and develop functional CTL responses following immunization with vaccinia virus. Next, we immunized chimeric animals with a DNA vaccine expressing HIV-1_{MN} envelope protein (pCEnv) and plasmids encoding CD80 or CD86 genes (pCD80 or pCD86). Using this model, we observed that in vivo transfection of only pCEnv and pCD86 could engineer non-bone marrow-derived cells such as muscle cells to prime and expand CTLs. This study suggests that CD86 and not CD80 plays a central role in the generation of the antigen-specific CTL responses. These results indicate that the strategy of engineering muscle cells to be more efficient APCs could be an important tool for the optimization of antigen-specific T cell-mediated immune responses in a pursuit of more rationally designed vaccines and immune therapies through the control of MHC class I restriction. This method of engineering nonhematopoietic cells to be more efficient APCs could be especially important in cases where antigen alone fails to elicit a CTL response due to poor presentation by the host APCs.

In addition to the B7 family of costimulatory molecules, the immunology of CD40 and its ligand has also been implemented to enhance vaccine potency. CD40 functions by interacting with CD40 ligand (CD40L) expressed on activated CD4+ or CD8+ T cells. The attachment of CD40L onto APCs, with or without T helper cells, has been shown to ''condition'' the APCs for antigen-specific CTL activation (143–145). In addition, their ligation has shown to enhance the expression of B7 costimulatory molecules on APCs, including dendritic cells (146–148). Therefore, the engagement of CD40 with its ligand becomes advantageous for activation of CTLs during an immune priming response. Accordingly, coimmunization of plasmids coding for β-galactosidase and CD40L has been reported to induce immune enhancement (149). Importantly, the addition induced enhancement of CTL responses without suppressing the development of antibody responses (150,151). Interestingly, coinjection of CD40L was revealed to be more effective than CD40 (152), indicating that expression of CD40L on muscle cells may also induce the ''licensing'' of APCs for the activation of CTLs. However, the possibility of direct transfection into antigen-specific infiltrating T cells cannot be eliminated, although T cells have not been yet established as an in vivo transfection target for DNA vaccines.

In light of these findings, we further investigated the strategy of engineering immune responses using additional costimulatory molecules (153). We coimmunized cDNA expression cassettes encoding intracellular adhesion molecule (ICAM)-1, lymphocyte function-associated antigen (LFA)-3, and vascular cell adhesion molecule (VCAM)-1, along with DNA immunogens, and analyzed the resulting antigen-specific immune responses. We observed that antigen-specific T cell responses can be enhanced by the coexpression of DNA immunogen and adhesion molecules ICAM-1 and LFA-3. Coexpression of ICAM-1 or LFA-3 molecules along with DNA immunogens resulted in a significant enhancement of Th cell proliferative responses. In addition, coimmunization with ICAM-1 (and more moderately with LFA-3) resulted in a dramatic enhancement of CD8-restricted CTL responses. Although VCAM-1 and ICAM-1 are similar in size, VCAM-1 coimmunization did not have any measurable effect on cell-mediated responses. Rather, these results imply that ICAM-1 and LFA-3 provide direct T cell costimulation. These observations were further supported by the finding that coinjection with ICAM-1 dramatically enhanced the level of IFN-γ and β-chemokines MIP-1α, MIP-1β, and RANTES produced by stimulated T cells. Through comparative studies, we observed that ICAM-1/LFA-1 T cell costimulatory pathways are independent of CD86/CD28 pathways, and they may synergistically expand T cell responses in vivo. Furthermore, these studies indicate that CD8+ effector T cells at the site of inflammation can regulate the level of effector function through the expression of specific chemokines and adhesion molecules (Fig. 7) (130,153). Therefore, the end-stage effector T cells in the expansion phase of an antigen-specific immune response could direct their destiny through coordinated expression and release of these molecules.

D. Cell Death to Enhance Immunogenicity

In addition to powerful signaling, dendritic cells often function as scavenger cells by engulfing and processing apoptotic bodies. For instance, immature dendritic cells phagocytose apoptotic bodies by employing the receptors alphavbeta5 integrin and CD36 (154–156). Subsequent engulfment of the apoptotic body by both immature and mature dendritic cells induces viral and tumor immunogens to activate MHC class I restricted

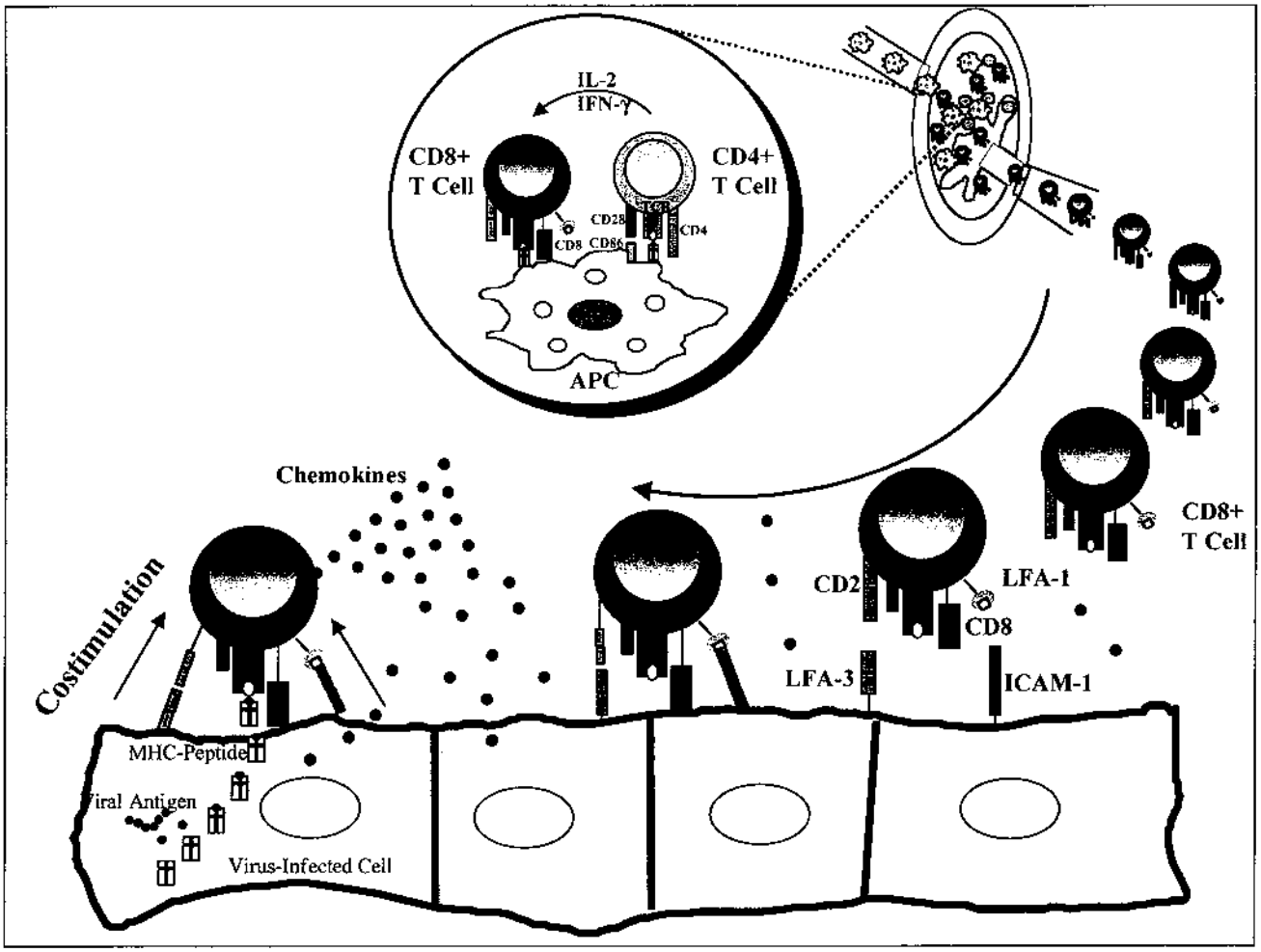

Figure 7 Regulation of CD8+ T cell expansion by adhesion molecules and chemokines in the periphery. Specific adhesion molecules and chemokines provide modulatory signals to CD8+ T cells in effector stage. This network of cytokine, chemokine, costimulatory molecules and adhesion molecules represents a coordinated regulation and maintenance of effector T cells in the periphery.

CD8+ CTLs (17,156). Interestingly, in vivo depletion of CD11c+ and CD8+ cells in mice fail to cross-prime antigens to prime naive T cells, specifying its essential role in inducing T cell activation (158). Both dendritic cells and macrophages have been shown to present apoptotic engulfed antigens, but the latter fails to activate naive T cells, which becomes a vital step in the activation of adaptive immunity (17,157). In addition, Rovere et al. and Ronchetti et al. demonstrated that there is a quantitative dependency on apoptotic bodies by dendritic cells in inducing the secretion of proinflammatory cytokines TNF-α and IL-1β both in vitro and in vivo, respectively (156,159). Hence, an optimum strategy to develop potent vaccines would necessitate the activation of dendritic cells and the packaging of immunogens in these apoptotic bodies for uptake. Appropriately, we have employed a novel strategy whereby immunogen constructs were coimmunized with the death cell receptor Fas. In theory, this model induces the expression of both the immunogen and Fas within the same muscle cells and apoptosis would materialize through the interaction of Fas with its ligand, possibly via T cell assistance. When these 2 constructs were coimmunized, there was a significant augmentation of immune responses as measured by enhanced CTLs and Th1 cytokines, including IFN-γ and IL-12 (160). In addition, implementing Fas as the apoptosis receptor may also provide a compounding effect on dendritic cell maturation, as Fas engagement possesses multiple functional roles. For instance, Fas not only contributes with the induction of cell death, but also stimulates the maturation of dendritic cells when engaged with its ligand (161). This is especially crucial, because direct in vivo transfection of dendritic cells has been proposed as a potential mechanism responsible for the induction of immune activation with respect

to DNA vaccines (18–20). More recent work by Sasaki et al. implemented mutant caspases to decrease apoptotic efficiency to aliquot ample time for immunogen expression, while still delivering apoptosis-mediated antigens to dendritic cells (162). This raises an interesting question because these mutant caspases decreased apoptotic efficiency in vitro by nearly 10-fold from the native caspase proteases (162). However, it is also currently understood that high quantities of apoptotic bodies are engulfed by scavenger macrophages and induce subsequent release of anti-inflammatory factors including IL-10, TGF-β, PGE2, and PAF (163,164). Accordingly, inflammation is observed when the clearance of apoptotic bodies becomes inefficient and results in their delayed removal from the surrounding environment (165). This results in the development of postapoptotic necrosis, which may function to deliver additional signals for proinflammatory developments and dendritic cell maturation (165,166). However, decrease in overall apoptotic quantity and potency with mutant caspases was able to provide enhanced immune response levels when compared with the more potent apoptotic signal (162). Therefore, it is also likely that delaying the expression of the apoptotic signal and/or working upstream of caspases may validate the requirement for the balance between immunogen expression vs. apoptotic stimuli (162). This reveals the necessity to delay the expression of apoptotic signals to maximize immunogen expression, while maintaining the potency of the death signal. It is also important to note that necrosis, not apoptosis, is traditionally the prime cell death mechanism by which inflammation and immune activation occurs (167). Interestingly, recent work suggests several models may be involved to induce immune activation through cell death. For instance, one model suggests that necrosis in conjunction with apoptosis delivers the maturation signal to dendritic cells (166,168). However, the role of postapoptotic necrosis as the stimulus is ruled out or insufficiently strong to deliver the maturation signal. Another model suggests that both primary necrotic and apoptotic cells are equivalent at inducing the maturation of dendritic cells and initiating immune activation (169). Therefore, either channel of cell death may be competent to stimulate the immune system for activation. Lastly, it is suggested that cell death or injury releases adjuvanting properties from the cytoplasm and is able to effectively stimulate immune activation (170). Therefore, components are actively secreted from dead cells that trigger the induction of immune responses. It is also evident that apoptosis and necrosis may function together to deliver immunogens to dendritic cells while inducing potent maturation signals (168). However, further studies are crucial to validate the precise roles that these cell death components may play in the generation of inflammatory responses with DNA immunizations.

VII. USE OF MOLECULAR ADJUVANTS IN PRIMATES

We sought to evaluate whether the enhancement of immune responses observed in mice with coimmunization with cytokine genes could also be achieved in rhesus macaques. DNA vaccines for HIV env/rev and SIV gag/pol alone were evaluated for their immunogenicity and compared with these vaccines, which also included IL-2, IFN-γ (Th1), or IL-4 (Th2) cytokine cDNA constructs (171). The cytokines dramatically enhanced seroconversion induced by the vaccines and appeared to modulate cellular responses as well, although more modestly. Vaccinated animals were challenged intravenously with SHIV IIIB. Half of the animals in the vaccine or vaccine plus Th1 cytokine groups exhibited protection from infection based on sensitive limiting dilution coculture, demonstrating a dramatic effect on viral replication of the vaccines tested. The protected animals were reboosted with SIV DNA vaccines (SIV and cytokine constructs) and were rechallenged intravenously with pathogenic SIV_{mac239}. All vaccinated animals were negative for viral coculture and antigenemia. In contrast, the control animals exhibited antigenemia by 2 weeks post challenge and exhibited greater than 10 logs of virus/10^6 cells in limiting dilution coculture (172). The control animal exhibited CD4 cell loss and developed SIV-related wasting within 14 weeks of high viral burden and subsequently failed to thrive. Vaccinated animals were virus negative and remained healthy. Although exact correlates of protection could include cellular responses, neutralizing antibody responses do not appear to correlate with control of viral replication and infection in these studies. These studies establish that multicomponent DNA vaccines can directly impact viral replication and disease in a highly pathogenic challenge system, thus potentially broadening our immunological weapons against HIV.

In an effort to enhance the clinical utility of the DNA-based PSA vaccine, we also examined the use of cytokine gene adjuvants to modulate vaccine-induced immune responses in animal models. We observed that pCPSA vaccine-induced humoral and cellular immune responses can be modulated through the coimmunization with cytokine genes in mice, and these enhancement effects on the PSA-specific cellular responses were extended in macaques. More specifically, coimmunization with IL-2 cDNA construct resulted in a significant enhancement of PSA-specific antibody responses in both mice and macaque models. In contrast, coinjection of IL-12 resulted in reduction of antibody responses in both models. In mice, the groups coimmunized with IL-2, IL-12, or IL-18 showed a dramatic increase in T helper cell proliferation over the results with pCPSA alone. These results support that further evaluation of this vaccination strategy to treat prostate cancer is warranted (173).

VIII. ADVANTAGES OF USING MOLECULAR ADJUVANTS

The overall objective of any immunization strategy is to induce specific immune responses that could protect the immunized individual from a given pathogen over his or her lifetime. One major challenge in meeting this goal is that the correlates of protection from an individual pathogen vary from

one infectious agent to the next. It would be a distinct advantage to design immunization strategies that can be ''targeted'' according to the correlates of protection known for the particular pathogen (Fig. 8). As summarized in Fig. 9, we observed that significant modulation was possible through the use of molecular adjuvants along with DNA vaccine constructs. This molecular adjuvant network underscores an important level of control in the induction of specific immune responses to tailor vaccination programs more closely to the correlates of protection, which vary from disease to disease. This type of fine control of vaccine and immune therapies was previously very difficult to obtain. Controlling the magnitude and direction of the immune response could be advantageous in a wide variety of vaccine strategies, including HIV-1. Much of the current literature supports that T cell-mediated responses are more critical for providing protective immunity against HIV infection (44,174,175). In such cases where T cell-mediated response is paramount, MCP-1, RANTES, or IL-12 genes could be chosen as the immune modulator to be codelivered with a specific DNA immunogen. However, for building vaccines to target extracellular bacteria, for example, MIP-1α, IL-4, IL-5, IL-10, or GM-CSF genes could be coinjected. In addition, in cases where both CD4+ T helper cells and antibodies play more important roles in protection, IL-2, IL-8, or GM-CSF could be codelivered. Furthermore, these genes can be combined with 1 or more additional cytokine or costimulatory genes to further control the immune responses. Additional studies in higher animal models such as the primates can further address potential risks and benefits of applying this genetic adjuvant network, ultimately leading to modulation of human diseases.

IX. RECOMBINANT PRIME/BOOST STRATEGIES IN PRIMATES

The generation of potent CTL responses through the utilization of DNA immunization has become an attractive approach against many pathogens, including HIV-1. Specifically, inefficient generation of antibodies possessing high avidity against different HIV strains have determined to be the downfall associated with humoral immune response dominant vaccines (176,177). However, an inverse correlation can be made between HIV viral load and CTL frequency, indicating its importance in both treatment and vaccine applications (174,178). In fact, virus-specific CTLs attribute significantly to the control of acute phase infection and denote the mechanism by which viral loads are controlled (179–181). Furthermore, studies on SIV infection models have also indicated that viremia of viral load could be effectively controlled by CD8+ T cells (181,182). As a result, recent applications have concentrated on maximizing the cellular arm of the immune system by generating potent CTLs to target and eliminate virally infected host cells. Previously through DNA vaccines, complete challenges were achieved against nonpathogenic AIDS viruses (118,120) and against pathogenic SHIV-89.6P (183). Collectively, DNA vaccines represent an effective means by which cellular responses can be raised against HIV. In view of this, one of the most promising DNA vaccine models is to manipulate the immune system by employing different prime/boost strategies. Moreover, studies by differing groups have garnered tremendous enhancement of CTLs by priming with DNA and boosting with attenuated viruses.

There is great intrigue with the immunology of recombinant virus boosting, as the order of application has shown to be crucial in generating potent enhancements. For instance, vaccine regimens in reverse (virus priming and DNA boosting) or merely DNA alone exhibit minimal amplification and fail to provide protective immunity against subsequent challenges (184–186). Historically, the order of application in attenuated prime/boost strategies has also exhibited similar results, as priming with a recombinant influenza virus and boosting with recombinant vaccinia resulted in enhanced CD8+ responses and protective immunity against malaria (187). It was proposed that perhaps the influences of these viruses to preferentially migrate CTLs to sites favored by the boosting virus may influence the overall potency of the vaccine (188). However, another study proposed that the immunogenicity may be correlated to the immunodominance of the boosting, as the antigen-specific memory responses from the priming may provide a greater focused isolation of the antigen of interest. Consequently, boosting with recombinant viruses concentrates the primed antigens from the recombinant and may enhance their amplification (186–188). This theory correlates with other reports, indicating that homologous prime/boosting with the same virus with heterologous antigens fails to proliferate antigen-specific CD8+ cells (189). Meanwhile, heterologous boosting with the modified vaccinia virus Ankara (MVA) and fowlpox viruses, while maintaining the same immunogen, provided significant enhancement of CTLs (190). It is also likely that the enhanced assembly of memory T cells that resulted from the primed immunization may augment the boost's proliferation by providing a larger memory T cell pool.

In addition to its efficacy, attenuated virus boosting specifically with MVA provides a resilient history for safety, as it was employed to vaccinate 120,000 humans during the smallpox eradication campaign (186,191). Furthermore, the inability of the virus to replicate in humans while maintaining efficient viral gene expression makes it a perfect component for recombinant boosting approaches (192,193). Cytokine studies of these viruses indicate an elevated degree of Th1 vs. Th2 responses, corresponding to the potent CTLs essential for an efficacious HIV-1 DNA vaccine (194,195). Further reports on the immunology of the MVA recombinant virus both in vitro and in vivo demonstrate that potent CTLs are generated as indicated by enhanced CD8+ populations and secretion of proinflammatory cytokines IL-6 and TNF-α (196–199). Consequently, the natural immune response of the virus correlates well with essential characteristics of vaccines by compounding the host's cellular responses.

To date, many different viruses have been studied to function as recombinant boosters for DNA vaccines, including the modified vaccinia Ankara, fowlpox, and the adenovirus

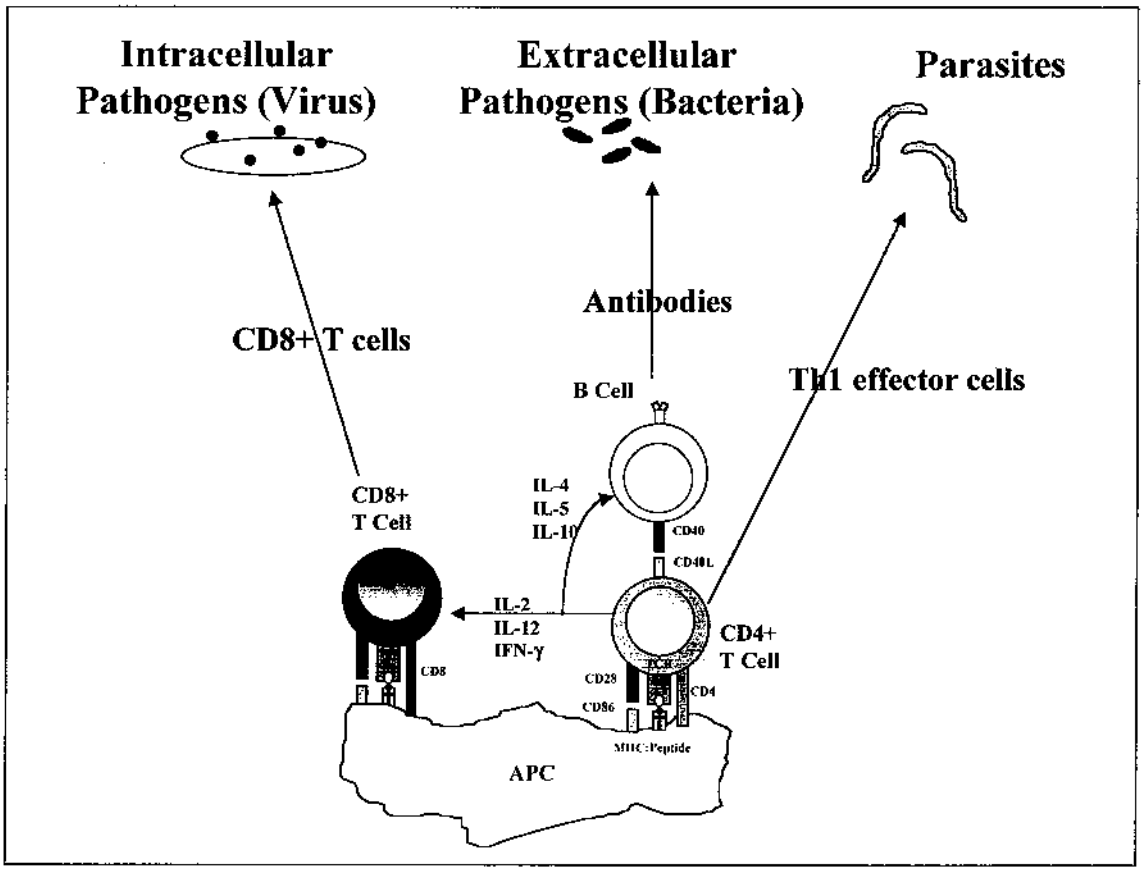

Figure 8 The potential utility of the molecular adjuvant network. Tailoring the induction of specific immune responses by vaccination programs against viral, bacterial, or parasitic diseases could be beneficial.

(200–205). Although most have reported potent CTLs and enhanced Th1-biased cytokine expression, the most promising are recent studies implementing the DNA/MVA boost scheme, as well as the DNA/Adeno5 model. The rhesus macaque from these studies garnered protective responses against subsequent pathogenic SHIV89.6P challenges by preventing clinical AIDS, while displaying low to no viral loads. The overall comparisons indicate that the most promising model is the Ad5 through survival of higher T cell counts and lower viral loads (205). However, the final judgments for these vaccines are premature until the evaluation of these models in human trials. This is especially of importance as previous challenged primates have been shown to eventually regress and develop clinical AIDS because of mutations within the gag dominant epitope (206). However, these challenges were conducted with the more pathogenic 89.6 SHIV model, and do not fully signify and mimic the in situ human HIV-1 infection setting.

X. CONCLUSION

DNA immunization holds great promise for providing safe and inexpensive vaccines for many infectious pathogens and cancer. The direct injection of foreign genes by genetic immunization has resulted in specific immune responses that exhibit characteristics of protective immunity against a number of infectious agents in a variety of animal models. For example, genetic vaccination cassettes targeting each of HIV-1's 3 major genes (env, gag, and pol), regulatory genes, and accessory genes have been developed and studied in small animals, primates, and humans. DNA vaccine constructs for cancer-targeting tumor-specific antigens have also been studied in a variety of animal models. Developing successful vaccines for HIV-1 or cancer will likely involve targeting multiple antigenic components to direct and empower the immune system. Such a collection of immunization cassettes should be capable of stimulating broad immunity against both humoral and cellular epitopes, thus giving a vaccine the maximum ability to deal with viral immune escape or tumor growth. DNA vaccines can be combined with other vaccines, including recombinant protein, poxvirus, adenovirus, and others, to further enhance initial immune responses. In addition, the potential of the molecular adjuvant coadministration to dramatically enhance and regulate the antigen-specific humoral and cellular immune responses induced by DNA immunogens represents important

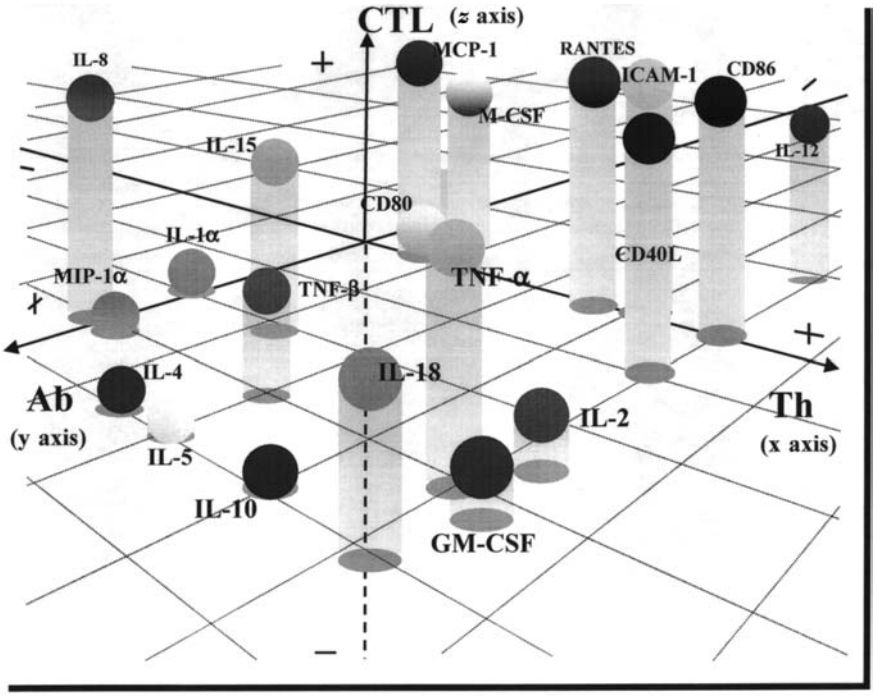

Figure 9 A summary of each cytokine coadministration effect on antibody (*y* axis), T helper (*x* axis), and cytotoxic T lymphocyte responses (*z* axis). Each cytokine is plotted on the 3-dimensional axis, according to its effects on the 3 modes of immune response. A summary of each molecular adjuvant coadministration effect on antibody (*y* axis), T helper (*x* axis), and cytotoxic T lymphocyte responses (*z* axis) is shown. Each adjuvant is plotted on the 3-dimensional axis, according to its effects on the 3 modes of immune response.

new avenues for vaccine and immune therapeutic exploration. Although further studies are warranted, these studies collectively support that optimized combinations of DNA expression cassettes coding for env, gag/pol, accessory proteins, costimulatory molecules, cytokines, chemokines, or other vaccine modulators may provide the basis for an effective vaccination strategy against infectious diseases and cancer.

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35

Cardiovascular Gene Therapy

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I. INTRODUCTION

Over that last quarter-century, numerous advancements have been made in the understanding of the molecular and cellular processes that lead to the development of atherosclerosis. The respective roles of the endothelium, inflammatory mediators, and thrombosis in the pathogenesis of vascular disease are beginning to be better understood. As more is learned of the initiation of atherosclerotic cardiovascular disease, new targets for systemic therapies are being discovered. Several classes of medications have been shown to be beneficial in preventing adverse cardiovascular events in patients with cardiovascular disease. These medications include platelet inhibitors (aspirin and thienopyridines), angiotensin-converting enzyme inhibitors, and 3-hydroxy-3-methylglutaryl-conenzyme A (HMG-CoA) reductase inhibitors (''statins'').

In conjunction with the improved understanding of the pathogenesis of vascular disease have been improvements in mechanical therapies for vascular disease. Surgical techniques have been perfected so that obstructed arteries can be effectively bypassed (as in coronary artery bypass grafting and lower- extremity bypass grafting), or be cleared of obstructive plaque (as in carotid endarterectomy). Revascularization strategies have since moved to less invasive endovascular techniques. Coronary arteries are routinely treated with metallic stents to improve myocardial blood flow and reduce ischemic symptoms. Stents are also routinely placed in iliac arteries for disabling claudication or critical limb ischemia, renal arteries for renovascular hypertension, and more recently, carotid arteries for the prevention of stroke. Percutaneous therapies are slowly supplanting surgical therapies as the treatment of choice for patients with cardiovascular disease, particularly in those patients with significant comorbidities limiting surgical options.

Ironically, while advancements in therapy have resulted in patients living longer with more severe cardiovascular disease, they have also resulted in a growing population of people who are no longer candidates for conventional therapies for their symptoms. These ''no-option'' patients live with severe angina, congestive heart failure, or disabling claudication/limb ischemia, and are becoming an increasingly larger part of our aging society. The fundamental problem in these patients is a deficiency in the blood supply to the myocardial and lower-extremity muscle beds due to severe, diffuse, and often totally occlusive vascular disease. The next ''holy grail'' in cardiovascular medicine is to stimulate the development of new vasculature to ischemic tissue (therapeutic angiogenesis) in order to improve blood flow, improve end-organ function, and relieve symptoms. Gene therapy may prove to be the most effective means of promoting therapeutic angiogenesis. Before reviewing gene therapy for cardiovascular disease, a description of vasculogenesis, angiogenesis, and angiogenic growth factors is warranted.

II. VASCULOGENESIS AND ANGIOGENESIS

In 1971, Folkman and colleagues published their pioneering work on growth factors, suggesting that the establishment and maintenance of a vascular supply is essential for growth of normal as well as neoplastic tissue (1). The establishment of a vascular supply occurs as a result of 2 main processes: vasculogenesis and angiogenesis. Vasculogenesis is the de novo in situ differentiation of endothelial cells (ECs) from mesodermal precursors in the embryo by association of endothelial progenitor cells (EPCs) or angioblasts and their subsequent reorganization into a primary capillary plexus 2. In contrast, angiogenesis is the formation of new blood vessels from

preexisting blood vessels. Angiogenesis is induced by the proliferation and migration of preexisting, fully differentiated ECs resident within parent vessels in response to stimuli such as hypoxia, ischemia, mechanical stretch, and inflammation (3,4). Angiogenesis can be a normal physiological process (e.g., as wound healing) or a pathological process (e.g., as in neoplasms and proliferative diabetic retinopathy).

Vasculogenesis was previously considered to be restricted to embryonic vascular development, while angiogenesis was believed to be responsible for both embryonic vascular development and postnatal neovascularization. Recent evidence, however, suggests that the basis for embryonic as well as therapeutic neovascularization likely encompasses both processes. Circulating CD34 antigen-positive EPCs were recently isolated from adult species and shown to differentiate along an endothelial cell lineage in vitro, thus constituting inferential evidence for the importance of rculating stem cells in angiogenesis (5). In addition, the demonstration that bone marrow-derived EPCs are increased in number in response to tissue ischemia, migrate and incorporate into foci of neovascularization in adult animals, and augment collateral development following *ex vivo* expansion and transplantation suggests that neovascularization in the adult involves both angiogenesis and vasculogenesis (6–8). Taiteshi-Yuyama et al. showed the potential of autologous stem cell transplantation to result in angiogenesis in patients with critical limb ischemia (9).

Arteriogenesis has been recognized as a mechanism that probably contributes to collateral vessel formation. A proportion of newly recognized medium-size arteries may be the result of proliferation of preexisting arteriolar connections into larger collateral vessels by remodeling (10). It is unknown whether such remodeling occurs as a direct result of growth factor modulation or as a flow-mediated maturation of these collateral conduits by a process of ''arteriolization'' of capillaries.

III. ANGIOGENIC GROWTH FACTORS

Although many cytokines have angiogenic activity, the best studied in animal models and clinical trials are vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).

A. Vascular Endothelial Growth Factor

The human VEGF proteins that have been identified to date are VEGF-1, VEGF-2 or VEGF-C, VEGF-3 or VEGF-B, VEGF-D, VEGF-E, and placental growth factor. All are encoded by different genes and localized to different chromosomes but share considerable homology. There are 4 isoforms of VEGF-1 that are the result of alternate splicing and are named according to the number of amino acids: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. These isoforms of VEGF show similar angiogenic potential in animal models (11), but differ in their solubility and heparin-binding capacity, accounting for differences in target cell binding. The principal cellular target of VEGF is the EC. There are 3 known endothelial-specific fms-like tyrosine kinases: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3. Hypoxia induces the formation of VEGF by the ECs and leads to up-regulation of VEGF receptors (VEGFRs) (12). VEGFR-1 generates signals that organize the assembly of ECs into tubes and functional vessels (13). VEGFR-2 is responsible for EC proliferation and migration (14,15). VEGFR-3 (Flt-4) principally mediates lymphangiogenesis (16).

VEGF possesses several features that facilitate gene transfer. First, VEGF contains a hydrophobic leader sequence that is a secretory signal sequence that permits the protein to be secreted naturally from intact cells, thus enabling a sequence of additional paracrine effects to be activated (17). Second, its high-affinity binding sites are exclusive to ECs; therefore, the mitogenic effects of VEGF are limited to ECs. This is in contrast to acidic and basic FGF, both of which are known to be mitogenic for smooth muscle cells and fibroblasts as well as ECs (18,19). Third, VEGF possesses an autocrine loop that is shared by most angiogenic cytokines and facilitates modulation of EC behavior. When activated under hypoxic conditions, the autocrine loop serves to amplify and thereby protract the response in ECs stimulated by exogenously administered VEGF. Furthermore, factors secreted by hypoxic myocytes up-regulate VEGFR expression on ECs within the hypoxic milieu. Such localized receptor expressions may explain the finding that angiogenesis does not occur indiscriminately, but rather is relatively limited to sites of tissue ischemia. Recently, an important additional role for VEGF has been described in augmentation of circulating EPC numbers documented in mice and humans following VEGF gene transfer (20–22). These EPCs have been shown to home into areas of myocardial ischemia.

B. Fibroblast Growth Factor

FGF is a family of 9 factors, including acidic FGF (FGF-1), basic FGF (bFGF or FGF-2), and FGF 3-9. Acidic FGF and basic FGF are the most extensively characterized members of the FGF family. FGFs are nonsecreted growth factors lacking a signal peptide sequence. The extracellular release of FGF is caused by cell death or damage. It binds to tyrosine kinase receptors via cell surface heparan sulfate proteoglycans, and as result, FGF is rapidly removed from the circulation and localized to cells and extracellular matrix. Although FGFs are potent EC mitogens, they are not EC specific and also serve as ligands for other cell types, including vascular smooth muscle cells and fibroblasts. At least 4 high-affinity FGF receptors have been identified and their cDNAs have been cloned. The FGFs, like VEGF, also stimulate EC synthesis of proteases, including plasminogen activator and metalloproteinases, important for extracellular matrix digestion in the process of angiogenesis (23).

Unlike VEGF, however, the common forms of FGF (FGF-1 and -2) lack a secretory signal sequence, and therefore, clinical trials of FGF gene transfer have required either modification of the FGF gene or use of another of the FGF gene family with a signal sequence (24–26).

IV. THERAPEUTIC ANGIOGENESIS

Angiogenic cytokines may be administered as recombinant protein or as genes encoding for these proteins. Given that both protein and gene delivery approaches have been relatively well tolerated thus far in clinical trials, ongoing investigations will determine the optimal preparation and delivery strategy for therapeutic neovascularization. Protein therapy remains the more conventional approach, and some investigators have indicated that this strategy is the closest to practical use. Nevertheless, recombinant protein is usually administered systemically and several issues limit its use. First, high plasma concentrations are required to achieve adequate tissue uptake to translate into a meaningful biological effect. This leads to higher potential for adverse effects. Second, recombinant human protein is difficult to produce and the costs are prohibitive.

Gene transfer allows for the high levels of sustained gene expression without provoking adverse host reactions. The efficiency with which the transgene is introduced and expressed into the target cell and the duration of transgene expression determines the success of gene transfer strategies. Transfer vectors facilitate cellular penetration and intracellular trafficking of the transgene, and local delivery systems deliver the vector to the vicinity of the target cells.

There are two major categories of gene transfer systems: viral and nonviral. The most commonly used viral vectors for gene transfer are adenovirus and retrovirus. The nonviral methods for gene transfer include introduction of naked DNA into the target area or the transfer of genetic material via a liposomal vehicle.

Hypoxia stimulates secretion of the angiogenic cytokines and also causes an increased expression of nitric oxide (NO) and VEGF receptors. Thus, ischemic muscle represents a promising target for angiogenic growth factor therapy. Striated and cardiac muscles have been shown to take up and express naked plasmid DNA as well as transgenes incorporated into viral vectors. Moreover, previous studies have shown that the transfection efficiency of intramuscular gene transfer is augmented more than 5-fold when the injected muscle is ischemic (27,28). Viral vectors may enhance transfection efficiency and thus yield higher levels of gene expression. In vitro and in vivo models have demonstrated that low-efficiency, but site-specific transfection (successful transfection in $<1\%$ of cells) with a gene (plasmid DNA) encoding for a secreted protein (e.g., VEGF) may overcome the handicap of inefficient transfection (29,30). By secreting adequate protein locally that translates into physiologically meaningful biological effects, therapeutic effects are achieved that are not realized by transfection with genes encoding for proteins that remain intracellular (e.g., bFGF). Furthermore, unlike viral vectors, plasmid DNA does not induce inflammation.

V. CLINICAL TRIALS OF VEGF PROTEIN AND GENE THERAPY

A. Peripheral Vascular Disease

The consensus statement of the European Working Group on Critical Limb Ischemia states that no medical treatment has been shown to alter the natural history of critical limb ischemia (31). In a large proportion of patients with critical limb ischemia, the distribution and extent of the arterial occlusive disease makes percutaneous or surgical revascularization impossible. In advanced stages of disease, quality-of-life measures are comparable to those patients with terminal cancer (32). Despite appropriate medical and surgical therapy, the unrelenting course of the disease ultimately leads to amputation. Of patients undergoing 1 amputation, 10% require a second amputation (33–36).

Despite the associated morbidity and mortality associated with amputation, it is often chosen as first-line therapy. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling. Significant research has focused on developing angiogenic therapies to provide novel approaches to the treatment of limb ischemia.

Preclinical studies have established proof of principle for the concept that the angiogenic activity of VEGF is sufficiently potent to achieve therapeutic benefit. After intra-arterial administration of recombinant VEGF protein, augmentation of angiographically visible collateral vessels and histologically identifiable capillaries were demonstrated in rabbits with severe, unilateral hindlimb ischemia (37). Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on other animal models, including rat and rabbit cornea, the chorioallantoic membrane, and the rabbit bone graft model (18,38). Intra-arterial gene transfer of $phVEGF_{165}$ in a human patient subsequently demonstrated angiographic and histologic evidence of angiogenesis (39).

Intra-arterial delivery, however, has several inherent limitations that could undermine successful growth factor transfer for critical limb ischemia. In the case of recombinant proteins, large doses of protein are necessary to exert a treatment effect in the face of rapid degradation by circulating proteinases. With naked DNA (i.e., DNA unassociated with viral or other adjunctive vectors), cellular uptake is virtually nil when the transgene is directly injected into the arterial lumen, presumably due to prompt degradation by circulating nucleases. In addition, the diffuse distribution of neointimal thickening and/or extensive atherosclerotic disease may limit gene transfer to the smooth muscle cells of the arterial media (40).

Preclinical studies of VEGF gene therapy were therefore designed to establish the feasibility of site-specific intramuscular gene transfer of VEGF in critical limb ischemia to promote therapeutic angiogenesis. Meaningful biological outcomes were observed following VEGF gene transfer of naked DNA by direct injection into skeletal muscle of ischemic rabbit hindlimbs, as evidenced by increased hindlimb blood pressure ratio, increased Doppler-derived iliac flow, enhanced neovascularity by angiography, and increased capillary density at necropsy (28,41).

Intramuscular gene transfer of 4000 μg naked plasmid DNA-encoding VEGF ($phVEGF_{165}$) was used to successfully accomplish therapeutic angiogenesis in patients with critical limb ischemia (42). Gene expression was documented by a transient increase in serum levels of VEGF monitored by en-

zyme-linked immunosorbent assay (ELISA). Meaningful clinical and physiological benefit was demonstrated by regression of rest pain and/or improved limb integrity, increased pain-free walking time, increased ankle-brachial index (ABI), newly visible collateral vessels by digital subtraction angiography, and qualitative evidence of improved distal flow by magnetic resonance imaging.

In a subsequent clinical trial in 55 patients (ages 24–84 years, $m = 56.7$ years) with ischemic rest pain ($n = 14$) or ischemic ulcers ($n = 41$) were treated with intramuscular injections of $phVEGF_{165}$. Evidence of clinical improvement was observed in 13 of 14 (72%) patients with rest pain alone and 26 of 41 (63%) patients with ischemic ulcers over a follow-up period of 4 to 36 months. For the total cohort of 55 patients, a favorable clinical outcome was achieved in 65.5%. Multiple logistic regression analysis identified rest pain and age <50 years as significant, independent predictors of a favorable clinical outcome. Diabetes, smoking, hyperlipidemia, hypertension, and $phVEGF_{165}$ dose were not predictors of clinical outcome (43). Complications in these patients have been limited to lower-extremity edema that develops in approximately one-third of patients 44. Edema was either self-limited or required a brief course of diuretic therapy, and it resolved approximately 1 to 2 months after gene transfer.

A similar treatment strategy was used in 11 patients with Buerger's disease presenting with critical limb ischemia, 9 of which were successfully treated with intramuscular $phVEGF_{165}$ (45). These patients had resolution of nocturnal rest pain and healing of foot and/or leg ulcers. The ABI increased by greater than 0.1, and newly formed collateral vessels were seen on MRA and serial contrast angiography.

Preclinical studies from our laboratory demonstrated that VEGF-2 could promote angiogenesis in a rabbit hindlimb ischemia model and stimulate the release of NO from ECs (46). Based on these preclinical studies, a randomized, double-blind, placebo-controlled, dose-escalating trial to investigate the therapeutic potential of VEGF-2 gene transfer in patients with critical limb ischemia (CLI) was recently completed. Forty-eight patients were enrolled between 1999 and 2000. These patients had Rutherford category 4/5 limb ischemia and no revascularization options. The dose of gene used ranged between 1.0 and 4.0 mg. Patients received 8 calf injections, were followed up weekly for 12 weeks and then monthly for 3 months, and visited at 9 and 12 months. Because from this study are currently being reviewed, the results are unavailable.

Recently, Nabel et al. published preliminary results of a phase I trial to evaluate the safety of an adenoviral vector encoding $VEGF_{121}$ in patients with disabling peripheral arterial diseases. Intramuscular injections of the adenoviral vector were performed in skeletal muscle of the lower limbs at sites of desired collateral formation. There was a favorable influence on lower-extremity endothelial function and flow reserve in 5 patients treated (47).

B. Prevention of Restenosis After Peripheral Angioplasty

In the adductor canal, the superficial femoral artery (SFA) is prone to stenosis and this represents one of the most common sites of peripheral arterial obstruction. Several postulates have inadequately explained this phenomenon. Percutaneous transluminal angioplasty (PTA) has been used widely and successfully to treat atherosclerotic obstructions in the peripheral and coronary circulations. However, high rates of restenosis following angioplasty of the SFA/popliteal artery continues to be a vexing, and consequently, expensive complication of this otherwise efficacious intervention. Although immediate procedural success for percutaneous revascularization of lesions in the SFA using conventional guide wires and standard PTA is well in excess of 90%, published reports have established that restenosis may complicate the clinical course of as many as 60% of patients undergoing PTA for SFA stenosis and/or occlusion. Previous strategies to limit the development of restenosis by nonmechanical means have not proved effective. Treatment strategies aimed at specifically restoring endothelial integrity have not been previously explored for restenosis prevention. Animal studies demonstrated that administration of mitogens, such as VEGF, which promote EC migration and/or proliferation, might achieve acceleration of reendothelialization and thereby reduce intimal thickening (48–51).

We therefore designed a phase 1, single-site, dose-escalating, open-label, unblinded gene therapy trial to accelerate reendothelialization at the site of PTA-induced endothelial disruption as a novel means to inhibit restenosis following PTA. The primary objective of this study was to document the safety of percutaneous catheter-based delivery of the gene encoding VEGF in patients with claudication due to SFA obstruction.

Arterial VEGF gene transfer has thus far been performed in 30 patients, 21 males and 9 females, with a mean age of 68 years. All patients had 2 or more cardiovascular risk factors. Gene expression was documented by a rise in plasma levels of VEGF. Peak plasma levels were recorded at a mean of 12 days following gene transfer. Mean claudication time increased from 2 min at baseline to 5 min up to 18 months post gene transfer. Prior to gene transfer, all patients were classified as Rutherford class 3. At 12 to 18 months following gene transfer, 15 patients were asymptomatic and 8 patients were class 1. After an initial improvement in 2 Rutherford classes following revascularization, 6 patients returned to class 3. One patient developed critical limb ischemia and required salvage therapy with intramuscular gene transfer of naked plasmid DNA encoding VEGF.

There was a significant and sustained improvement in ABI, post gene transfer compared with baseline. Prior to gene transfer, the mean ABI was 0.70, increased to 0.92 at 18 months after gene transfer, and was sustained at 0.91 at 48 months after gene transfer. SFA stenosis in 24 patients dropped from a mean of 94% at baseline to 30% at an average of 9 months following gene transfer. These results were supported by intravascular ultrasound (IVUS) findings at the time of follow-up angiography. Six patients had evidence of restenosis at angiography performed 6 to 12 months following gene transfer. Target vessel revascularization was required in all 6 patients. Histology from 3 of 4 patients undergoing directional atherectomy at the time of repeat revascularization for restenosis demonstrated active smooth muscle cell proliferation and

high levels of proliferating cell nuclear antigen, indicating extensive proliferative activity.

Thus, in the 30 patients that have been treated with arterial VEGF gene transfer for prevention of restenosis, VEGF expression has been documented by ELISA. At 48 months' follow-up, 6 of 30 patients (20%) required target vessel revascularization for angiographic and ultrasound evidence of restenosis. When compared with historical controls, at 6 months, restenosis rate was 20% in the gene transfer group, 29% in patients undergoing brachytherapy, and 55% in patients undergoing percutaneous angioplasty alone (52). This preliminary study has suggested that gene therapy designed to accelerate reendothelialization at the site of PTA-induced endothelial disruption can be safely performed. Importantly, no evidence of accelerated atherosclerosis or an increase in the restenosis rate was observed following gene transfer.

C. Myocardial Ischemia

For patients in whom antianginal medications fail to provide sufficient symptomatic relief, other interventions such as angioplasty or bypass surgery may be required. Although both types of intervention have been shown to be effective for various types of patients, a considerable group of patients may not be candidates for either intervention due to the diffuse nature of their coronary artery disease. Moreover, there are many patients in whom recurrent narrowing and/or occlusion of bypass conduits after initially successful surgery has left the patient again symptomatic with no further option for conventional revascularization.

For the purposes of myocardial angiogenesis, $VEGF_{165}$ recombinant protein has been administered via a wide variety of routes. Phase I studies of both intracoronary and intravenous injection of $VEGF_{165}$ recombinant protein in patients with symptomatic, inoperable coronary artery disease revealed encouraging improvements in anginal status as well as both rest and stress nuclear perfusion studies (53–55).

The Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis (VIVA) study was a phase 2, double-blind, placebo-controlled, multicenter, dose-escalating trial of patients with angina and viable myocardium who were not optimal candidates for percutaneous or surgical revascularization. Patients randomized to the treatment group received intracoronary injections and 3 intravenous infusions injections of VEGF-1 protein (56–59). The doses used were 17 or 50 μg/per minute via intracoronary route for 20 min and intravenously for 4 h on days 3, 6, and 9. One hundred and fifteen patients received the recombinant VEGF and 63 patients received the placebo. Patients were followed up at 60 days, 120 days, and 1 year. At 60 days, there were similar increases in exercise time for both treatment and placebo group (approximately 45 sec). Similarly, there were similar decreases in angina and quality of life, and no change in perfusion studies. At 120 days, patients who received the high dose had a decrease in angina grade and a trend to increased exercise time. At 1 year follow-up, there were no statistical difference in the clinical and measured parameters, but there was a trend toward decrease angina class in patients receiving the VEGF-1 recombinant protein. Angiographic and single positron emission computed tomography (SPECT)–Sestamibi scan did not show any significant change in any group.

The VIVA trial demonstrated that hypotension was a significant limitation in the dose of recombinant VEGF that could be administered intravasculary. This complication of recombinant VEGF therapy was also seen in animal experiments performed in our laboratory using recombinant human VEGF ($rhVEGF_{165}$). Hypotension after systemic administration of recombinant VEGF is believed to be mediated by VEGF-induced release of NO (60). Similar results were reported from other groups using intracoronary injection in the pig and dog (61,62). Other routes of administration of VEGF (intramyocardial, periadventitial, and intravenous) have shown limited efficacy (61,63,64) likely due to an inability to administer a sufficient dose of the protein.

Accordingly, it has been hypothesized that local expression of VEGF for a protracted period of 2 to 3 weeks via gene transfer might circumvent the problem of symptomatic hypotension, yet still achieve a reduction in myocardial ischemia. VEGF gene transfer for myocardial ischemia has been performed in animal models both by using adenovirus vectors as well as by the administration of naked plasmid DNA. Intramyocardial injection of adenovirus encoding $VEGF_{121}$ via thoracotomy in a pig ameroid model improved collateral perfusion and function (65,66). Intracoronary adenoviral gene delivery produced much lower gene and VEGF levels in the myocardium with poor localization (66). Pericardial delivery of adenovirus encoding $VEGF_{165}$ in a dog model did not increase collateral flow (67).

Our center initiated a phase 1, dose-escalating, open-label clinical study to determine the safety and bioactivity of direct myocardial gene transfer of $phVEGF_{165}$ as sole therapy (i.e., without angioplasty, stenting, or bypass graft surgery) for myocardial ischemia. Patients with stable exertional angina refractory to medical therapy, areas of viable but underperfused myocardium on perfusion scanning, and multivessel occlusive coronary artery disease were selected. Preliminary results of this trial suggested that safe and successful transfection could be achieved by this method with a favorable clinical effect (68,69). Thirty patients with mean age of 63 years were selected. Twenty-nine out of 30 patients had coronary artery bypass graft (CABG) surgery, and all had suffered myocardial ischemia. All received $phVEGF_{165}$ administered by direct myocardial injection in 4 aliquots of 2.0 mL via a ''minithoracotomy''; total dose 125 μg ($n = 10$), 250 μg ($n = 10$), 500 μg ($n = 10$). By using a stabilizing device that facilitates vascular anastomosis during beating heart bypass an immobile field for intramyocardial injection was ensured. Continuous transesophageal echocardiographic monitoring was performed throughout the procedure to monitor development of wall motion abnormalities associated with injections and ensure that plasmid DNA was not injected into the LV cavity (70). No perioperative complications occured. There was no evidence of myocardial damage by cardiac enzyme analysis and patients maintained left ventricular function. Gene expression was documented by a transient but significant increase in plasma levels of VEGF monitored by ELISA

assay. All patients experienced marked symptomatic improvement and/or objective evidence of improved myocardial perfusion. At 360 days, 15 of 30 patients were free of angina. Specifically, sublingual nitrate use fell from 60 per week to 3 per week at day 360, accompanied by a significant reduction in episodes of angina from 56 per week to 4 per week at day 360. Exercise time for the group at 360 days had increased by 98 sec and exercise time to angina increased by 2.5 min over baseline. There were 2 late deaths (4.5 and 28.5 months), and 1 patient underwent a cardiac transplant at 13 months (69). Evidence of reduced ischemia on SPECT–Sestamibi myocardial perfusion scanning was documented in 22 of 29 patients with a significant reduction in both stress and rest mean perfusion/hemia score at day 60. In addition, 22 of 29 patients (76%) improved by 2 Canadian Cardiovascular Society (CCS) angina classes at 12 months, and 20 of 28 patients (71%) improved by equal or more than 2 CCS classes at 2 years.

It is intriguing to note that not only defects observed in the perfusion scans with pharmacological stress, but also those observed at rest, improved post gene transfer; sequential SPECT scans recorded before and after gene transfer demonstrated partial or complete resolution of fixed defects in 4 (33%) and 5 (43%) patients, respectively, in whom defects were present on the initial rest image. This is consistent with the notion that these preexisting defects constitute foci of hibernating viable myocardium that have resumed or improved contractile activity as a result of therapeutic neovascularization (71–73). This observation was supported by the findings of electromechanical mapping used in the final 13 consecutive patients. Resting perfusion defects on the SPECT images corresponded to areas with ischemic characteristics (reduced wall motion with preserved viability) on the endocardial maps. Foci of ischemia were identified preoperatively in all patients with significant improvement in these endocardial wall motion abnormalities at 60 days post gene transfer (74). This study provides the first evidence for a favorable clinical effect of direct myocardial injection of naked plasmid DNA encoding for VEGF as the sole therapeutic intervention.

A similar favorable experience was seen in an open-label, dose-escalating, multicenter clinical trial of VEGF-2 plasmid DNA in 30 patients with end-stage coronary artery disease and refractory class III or IV angina. Twenty-four male patients and 6 female patients with a mean age of 61 were selected. All had previous CABG, and all had sustained 1 to 2 episodes of myocardial ischemia. Their medical regimen included more than 2 antianginal medications. In all patients, there were no procedural adverse events, although there was 1 death 20 h after surgery. At 12 months following gene transfer, the mean number of anginal episodes and nitrate tablets consumed per week decreased significantly. By day 90, 21 of 30 (70%) patients improved by more than 2 CCS angina classes with an additional 4 patients (25 of 29 patients or 86%) with similar improvement at 360 days. The mean duration of exercise also increased by more than 2 min (Vale, et al., 2000, unpublished data).

The other reported study of direct myocardial VEGF gene transfer was with adenoviral-assisted $VEGF_{121}$ injection to patients undergoing bypass graft surgery ($n = 15$), and as sole therapy via minithoracotomy ($n = 6$). Symptoms and exercise duration improved in both bypass surgery and sole therapy groups, but stress-induced nuclear perfusion images remained unchanged. The data in this study are consistent with the concept that adenovirus $VEGF_{121}$ appears to be well tolerated in patients with advanced coronary disease.

The recently reported Efficacy and Safety of Gene Therapy in Patients with Advanced Coronary Artery Disease and No Options for Revascularization (REVASC) trial is the largest clinical trial in humans to date evaluating the efficacy of adenovirus mediated VEGF ($AdVEGF_{121}$) gene therapy for myocardial ischemia (75). In this phase 2, randomized, multicenter trial, 67 patients were randomized to receive $AdVEGF_{121}$ administered by direct intramyocardial injections via a limited thoracotomy or to continue with optimal medical management. At 26 weeks, time to ischemia on treadmill testing was significantly increased in the $AdVEGF_{121}$ vs. the medical therapy group ($P = 0.024$). There were significant improvements in anginal status as well as in several domains of the Seattle Angina Questionnaire in the $AdVEGF_{121}$ compared with the medical therapy group as well. Although these data are encouraging, there were 4 patients in the $AdVEGF_{121}$group that suffered cardiac complications due to the thoracotomy.

Each of the aforementioned studies has 1 major limitation: the need for thoracotomy for VEGF gene transfer. Thoracotomy has a small but real complication risk that can lead to substantial morbidity, particularly in patients with significant comorbidities. Furthermore, the strategy of gene therapy alone administered via a minithoracotomy does not permit randomization against placebo (untreated controls) or clinical testing of alternative dosing regimens, including multiple treatments.

Recent studies have suggested that a less invasive approach to gene transfer using a catheter-based delivery of naked plasmid $VEGF_{165}$ and VEGF-2 is effective in the pig (76). This less invasive approach to intramyocardial gene transfer has been shown to achieve suitable gene expression (76–79). Catheter-based myocardial gene transfer is performed using a previously described navigation system and catheter mapping technology (NOGA™) integrated with an injection catheter (Biosense-Webster, Warren, NJ), the distal tip of which incorporates a 27G needle to inject plasmid into the myocardium. To determine the safety and feasibility of catheter-based gene transfer, Vale et al. used this system to deliver naked plasmid VEGF to the myocardium of normal and ischemic swine (77). Results with methylene blue suggested safe, reliable, and reproducible targeting of endocardial sites. Injection of a reporter gene (pCMV-nls*LacZ*) demonstrated peak β-galactosidase (β-gal) activity in the target area with low-level to negligible activity seen in areas remote from the injection sites, suggesting relatively localized gene transfer. β-gal activity was greater in ischemic versus nonischemic myocardium indicating enhanced gene transfer in ischemic myocardium. Similar findings were demonstrated by a study using adenoviral-assisted gene transfer of a reporter gene (80). These results established that percutaneous myocardial gene transfer could be successfully achieved in normal and ischemic myocardium in a relatively site-specific fashion without significant morbid-

ity or mortality. The mapping capabilities of the NOGA system used in this study were useful for demonstrating that gene expression could be directed to predetermined left ventricle sites. This technique clearly may be advantageous for avoiding gene transfer to sites of myocardial scar, as well as relocating the tip of the injection catheter to areas of myocardial ischemia (or hibernating myocardium) where gene transfer will be optimized.

Subsequently, we initiated a pilot study of percutaneous, catheter-based VEGF-2 DNA gene transfer or a sham procedure guided by the NOGA mapping system in 6 patients with nonrevascularizable symptomatic myocardial perfusion (81). VEGF-2-transfected patients reported significant reduction in weekly anginal episodes and nitrate tablet consumption at 12 months post gene transfer. In contrast, although blinded patients randomized to the control group reported an initial reduction in these parameters, this changed clinical profile was not sustained past 30 days, suggesting that the continued reduction in angina in the VEGF-2-treated group was not a placebo effect. The symptomatic improvement was again accompanied by objective evidence of improved myocardial perfusion by both SPECT–Sestamibi perfusion scanning and electromechanical mapping (81). Although the clinical findings of this pilot trial concerning efficacy are similarly encouraging, the number of patients and the single-blind design preclude firm conclusions in this regard.

Consequently, a multicenter randomized, double-blind, placebo-controlled trial of catheter-based VEGF-2 gene transfer was initiated and results recently published. (82). Nineteen patients with chronic myocardial ischemia not amenable to percutaneous or surgical revascularization were randomized in a double-blind fashion to receive 6 injections of placebo or phVEGF-2. It was planned that 27 patients were to be randomized to receive gene or placebo in a 2:1 ratio. However, the study was interrupted by the Food and Drug Administration after 19 patients had been enrolled. Twelve patients received the gene product and 7 patients were randomized to placebo. A total of 114 injections were delivered through a steerable deflectable 8-french catheter with a 27-gauge needle guided by LV electromechanical mapping (NOGA). Perioperatively, there were no hemodynamic alterations, sustained arrhythmias, myocardial infarction, or ventricular perforation. There was a significant improvement in the CCS angina class at endpoint analysis at 12 weeks. Other endpoint analysis studied, including change in exercise duration, functional improvement in CCS by more than 2 classes, and Seattle Angina Questionnaire data showed strong trends favoring efficacy of phVEGF-2 compared with placebo treatment.

These preliminary experiences thus suggest that it is feasible to supplement or potentially replace currently employed operative approaches with minimally invasive techniques for applications of cardiovascular gene therapy designed to target myocardial function and perfusion. Such an approach may have at least 3 advantages compared with an operative approach. First, it potentially allows more selective delivery of the transgene to targeted ischemic zones, including sites that are less accessible by a minithoracotomy. Second, because it obviates the need for general anesthesia and operative dissection through adhesions resulting from prior surgery, the transcatheter approach facilitates placebo-controlled, double-blind testing of myocardial gene therapy. Third, the intervention can be performed as an outpatient procedure and repeated, if necessary.

D. Prevention of In-Stent Restenosis

Drug-eluting stents are expected to revolutionize interventional cardiology by reducing the incidence of in-stent restenosis from 30% to nearly 10%. However, the agents that have been studied to date as candidate drug coatings delay endothelial recovery. Prior studies have suggested that acceleration of endothelial cell formation following coronary stenting may attenuate the restenosis process. VEGF is a potent stimulator of endothelial cell recovery, and it has been hypothesized that a coronary stent coated with VEGF may have beneficial effects on prevention of stent thrombosis and prevention of in-stent restenosis. This hypothesis has been tested in a randomized fashion in the rabbit model of atherosclerosis (83). Fifty-four rabbits were treated with either an uncoated coronary stent or a stent coated with phVEGF-2 plasmid. At 3 months, the rabbits treated with the gene-coated stents had significantly larger lumen cross-sectional areas and a significantly smaller percent cross-sectional narrowing. In addition, there were increased numbers of EPCs and increased EC recovery (as assessed by NO production) in the rabbits treated with the gene-coated stents. Currently, a phase 1 trial is being designed for evaluating the safety of phVEGF-2-coated stents in humans. Alternative strategies at preventing restenosis are also being evaluated, including using a specialized infiltration catheter to directly deliver plasmid VEGF into coronary plaque prior to stenting.

VI. CARDIOVASCULAR TRIALS OF FGF PROTEIN AND GENE THERAPY

A. Peripheral Vascular Disease

Several investigators have shown improvements in muscle perfusion in animal models of hindlimb ischemia using recombinant FGF (84–86). The safety of intra-arterial bFGF administration in patients with intermittent claudication was recently demonstrated (87). In this phase I, double-blind, placebo-controlled clinical trial there was improvement in calf blood flow by strain gauge plethysmography in bFGF-treated patients at 6 months compared with controls. To date, the TRAFFIC study is the only clinical trial testing the efficacy of recombinant FGF in humans with peripheral vascular disease (88). A total of 190 patients with intermittent claudication were randomized to receive placebo, a single dose of FGF-2 (30 mcg/, or 2 doses of FGF-2. There was a trend toward patients receiving FGF-2 having increases in peak walking time at 90 days (0.60 min in placebo vs. 1.77 min in single-dose FGF-2 vs. 1.54 min in double-dose FGF-2, $p = 0.075$). There was no significant difference in adverse events between the 3 groups. The authors concluded that the increase in walking

time in patients receiving FGF-provided evidence that recombinant FGF-2 resulted in angiogenesis.

The first clinical trial in human subjects with peripheral vascular disease using FGF gene therapy was conducted on 51 patients with ischemic rest pain or tissue necrosis (89). This was a phase 1 evaluation of naked plasmid DNA encoding for FGF-1 (NV1FGF) administered via intramuscular injection. These patients were deemed to have severe obstructive lower-extremity vascular disease not amenable to mechanical revascularization. Patients received either escalating single dose or escalating double dose of NV1FGF. Overall, intramuscular injection of NV1FGF was well tolerated. Although there were adverse events, none were believed to be due to NV1FGF. Measurements of serum and plasma FGF-1 were made to assess gene expression. The distribution of plasmid in the plasma was limited, presumably due to destruction by endogenous endonucleases. There was no increase in serum FGF-1 levels. In the first 15 patients with completed 6-month follow-up, there was a significant reduction in pain and ulcer size as well as an increase in transcutaneous oxygen pressure compared with pretreatment values. There was also a significant increase in the ABI in these patients. This study confirmed the safety of NV1FGF for the treatment of inoperable lower-extremity peripheral vascular disease. The encouraging clinical results, however, need to be confirmed in larger, double-blind, placebo-controlled trials.

Presently, phase 1/2 clinical trials are ongoing in Europe to evaluate the safety and potential efficacy of FGF-4 delivered via an adenovirus vector (Ad5-FGF4) for the treatment of peripheral vascular disease. These are double-blind, placebo-controlled trials that will enroll up to 130 patients at 10 sites across Europe.

B. Myocardial Ischemia

A series of animal experiments have demonstrated that intracoronary and local delivery of FGF improves myocardial perfusion and function and increases collateral flow in myocardial ischemia (90–93). Several phase 1 trials have been performed to evaluate the safety of recombinant FGF-1 and FGF-2 for therapeutic angiogenesis in patients with myocardial ischemia (94–99). Each of these small studies showed reductions in anginal status, need for antianginal medications, and improvements in nuclear perfusion scanning. These studies, however, were limited by the fact that these patients were all scheduled for CABG surgery. Therefore, any potential applicability of these approaches would be limited to patients able to undergo thoracotomy. Intravenous and intracoronary administration of recombinant FGF-2 has also been evaluated in phase I studies of patients with symptomatic coronary artery disease with mixed results (100–102). Intravenous injection of recombinant FGF-2 was associated with improvements in anginal status, exercise time, and left ventricular function. However, intracoronary administration resulted in no change in exercise time or time to ischemia. Several patients experienced persistent hypotension for up to 3 days, conduction system disturbances, thrombocytopenia, and proteinuria.

The FIRST trial was a phase 2 that randomized 337 patients with inoperable coronary artery disease to receive either placebo, or 1 of 3 doses of intracoronary recombinant FGF-2 (103). The results of this trial did not show dramatic improvements in objective endpoints in patients receiving intracoronary FGF-2. The 90-day exercise time and stress nuclear perfusion results were not significantly different between FGF-2-treated patients and placebo-treated patients. There was trend toward improved anginal status in patients receiving intracoronary FGF-2, particularly in older and more symptomatic patients.

Results of these phase 1 and 2 trials again point to the potential shortcomings of recombinant protein therapy for therapeutic angiogenesis. FGF gene therapy in animal models of coronary ischemia has shown promise for therapeutic angiogenesis. In a chronic coronary occlusion animal model, human FGF-5 carried by an adenovirus vector (Ad5-FGF5) administered by intracoronary infusion resulted in sustained production of growth factors at 12 weeks, effective development of coronary collaterals, and relief of stress-induced ischemia (25,104).

Thus far, only 1 clinical trial in humans using FGF gene therapy for myocardial ischemia has been reported. Grines et al. conducted the Angiogenic Gene Therapy (AGENT) trial in which 79 patients with chronic stable angina (CCS class II and III) were randomized to receive placebo or 1 of 5 escalating doses of Ad5-FGF4 in a double-blind fashion (105). The Ad5-FGF4 was administered by a single intracoronary injection. Overall, patients receiving Ad5-FGF4 tolerated the infusion well with few immediate adverse events. One patient developed a fever during the first day after virus transfer and 2 patients had minor, self-limited elevations of liver enzymes. There were no significant differences in adverse events in patients receiving placebo and Ad5-FGF4 at a mean of 311 days of follow-up. Overall, patients receiving Ad5-FGF4 experienced a trend toward increased exercise times at 4 weeks. The trial prespecified a subgroup analysis in the 50 patients with baseline exercise times less than 10 min. There was a significant improvement in exercise time in this more symptomatic subgroup of patients in those who received Ad5-FGF5 (1.6 min vs. 0.6 min, $P < 0.01$). These data suggest that the intracoronary infusion of Ad5-FGF4 is safe and may be effective at improving hard clinical endpoints in patients with myocardial ischemia. Interestingly, in this study, patients enrolled were not ''no-option'' patients. The effects of intracoronary Ad5-FGF5 may be more dramatic in no-option patients. The true clinical value of this approach will be evaluated in future clinical trials designed to assess hard clinical endpoints.

VII. SAFETY CONCERNS REGARDING CARDIVASCULAR GENE THERAPY

Given that the inhibition of angiogenesis may be of value in the treatment of neoplasms, concern has been raised that the administration of angiogenic growth factors could lead to development of tumors. There are neither in vitro nor in vivo

data to suggest that either VEGF or FGF increases the risk of neoplastic growth and/or shmetastases, although longer-term follow-up will be required to address this issue in clinical trials. In our own experience with 88 subjects who have undergone VEGF gene transfer for CLI, the cumulative 7-year incidence of cancer has been limited to 2 patients with bladder cancer and 1 with liver and brain metastases from unknown primary (106). It was interesting to note that in the VIVA trial that there was a greater incidence of tumors in the placebo group compared with the VEGF group. This highlights the fact that the age group receiving such therapy will develop some unrelated tumors. Due to the theoretical risk of neoplastic growth, one must be vigilant about the possibility of cancer in patients treated with these angiogenic growth factors. In addition, concerns regarding the development of angiomata were raised in studies involving mice or rats treated with transduced myoblasts or supraphysiologic doses of plasmid DNA, respectively. Importantly, no other preclinical or clinical reports, including those using adenoviral vectors, have described this complication (107,108).

It is theoretically possible that VEGF may exacerbate proliferative and/or hemorrhagic retinopathy in patients with diabetes in view of the high VEGF levels demonstrated in the ocular fluid of patients with active proliferative retinopathy leading to loss of vision (109). To date, this adverse effect of therapeutic angiogenesis has not been observed. The local delivery of naked plasmid DNA encoding for VEGF-1 or VEGF-2 to more than 100 patients (one-third with diabetes and/remote retinopathy) treated at our institution with up to 4-year follow-up did not effect the visual acuity or fundoscopic findings as evidenced by serial funduscopic examinations pre- and post gene transfer by an independent group of retinal specialists.

Experiments in transgenic mice engineered to overexpress VEGF ± angiopoietin have demonstrated lethal permeability-enhancing effects of VEGF (110). However, even though VEGF has been reported to cause local edema, which manifests as pedal edema in patients treated with VEGF for CLI, it responds well to treatment with diuretics (44). As previously described, hypotension has been observed in therapies with recombinant proteins, particularly when used systemically and in higher doses (111,112). This is believed to be due to the fact that VEGF up-regulates NO synthesis. This complication, however, has never been described following gene transfer in either animals or humans (113,114).

Moulton et al. observed that when hypercholesterolemic, apolipoprotein E- deficient mouse models were treated with inhibitors of angiogenesis (endostatin or TNP-470), there were significant regression of plaque areas and inhibition of intimal neovascularization (115). This study with several other studies raised concern regarding the potential for VEGF and other proangiogenic therapies to promote atherosclerosis (92, 116,117). However, data available from 4 separate animal studies and 2 clinical studies of human subjects fail to support the notion that accelerated atherosclerosis is a likely consequence of administering angiogenic cytokines (48–51, 118,119). The outcome is quite the opposite, in that administration of VEGF led to a statistically significant reduction in intimal thickening due to accelerated reendothelialization, thereby refuting the notion that acceleration of atherosclerosis will be a consequence of VEGF-induced stimulation of angiogenesis.

VIII. CONCLUSIONS

The preliminary effectiveness of gene therapy for therapeutic angiogenesis in patients with critical limb and chronic myocardial ischemia are both encouraging and promising. The different trials using angiogenic protein and transgenes encoding for VEGF and FGF attest to the safety and effectiveness of these strategies. The current clinical strategies employed for CLI and chronic myocardial ischemia constitute an extrapolation from initial applications of gene transfer to animal models with limb ischemia. These results, however, likely have generic implications for strategies of therapeutic neovascularization using alternative candidate genes, vectors, and delivery strategies. Preclinical data supporting the use of other VEGF-1 isoforms (120), as well as other VEGF genes (121), has been previously reported as having preclinical studies using FGF (25,122); all these are being actively studied in ongoing clinical trials. Furthermore, the relative merits of gene transfer vs. recombinant protein administration remain to be clarified.

The otherwise negative primary endpoint results of the VIVA and FIRST studies using intracoronary ± intravenous protein administration underscore the concern that the pharmacokinetics of recombinant protein administered into the vascular space may lead to inadequate local delivery of angiogenic growth factor within the ischemic myocardium. Additional investigations comparing doses of recombinant protein and routes of delivery will be required to resolve this issue. Until these studies are complete, the ideal method of achieving therapeutic angiogenesis remains unknown. In addition, results of phase 1 studies, designed by definition to assess safety, must be interpreted with caution. Typically, the number of patients enrolled in such trials is relatively small, and for those lacking a control group, a placebo effect cannot be excluded. For studies in which recombinant protein or gene is administered in conjunction with conventional revascularization, it may be difficult to determine the relative contributions of the angiogenic agent vs. bypass surgery to the symptomatic response.

It is clear, however, that site-specific VEGF gene transfer can be used to achieve physiologically meaningful therapeutic modulation of vascular disorders and specifically that IM injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia. Of note, there was no evidence of immunological toxicity neither in our intra-arterial animal studies, nor our human clinical experience using naked plasmid DNA encoding for VEGF. Furthermore, at this early stage of clinical trials into myocardial gene therapy, it has been shown that direct myocardial gene

transfer using different doses of naked plasmid DNA encoding for $VEGF_{165}$ and VEGF-2, as well as intracoronary FGF-5 carried by an adenovirus vector, can be performed safely with augmentation of myocardial perfusion. The catheter-based delivery of plasmid DNA is an attractive and safe option. In terms of safety, no operative complications and no aggravated deterioration in eyesight due to diabetic retinopathy (123) have been observed in patients treated with $phVEGF_{165}$ gene transfer. With specific regard to mortality, it should be noted that the cumulative mortality for the 85 patients with class 3 or 4 angina undergoing operative or percutaneous naked DNA gene transfer of VEGF-1 or VEGF-2 has been 3 of 85 or 3.5% at up to 33 months follow-up. This compares favorably with an average 11% to 13% 1-year mortality for a similar group of almost 1000 patients receiving laser myocardial revascularization or continued medical therapy in 5 contemporary controlled studies (124–128). Ongoing clinical studies will determine the potential for neovascularization gene therapy to be performed by nonsurgical, catheter-based delivery, although early results are encouraging from a therapeutic standpoint.

For the most part, clinical studies of therapeutic angiogenesis have been restricted to patients with myocardial or limb ischemia who have no other options. Although this is the group to target in the near future, it is not difficult to foresee a time when a significant populations of patients who undergo bypass surgery but are not optimal candidates for that procedure may be eligible for therapeutic angiogenesis. The latter might be performed at an earlier stage of disease, and the potential for repeat treatment may translate in a greater possibility of a successful outcome.

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36

Selectively Replicating Adenoviruses: Clinical Experience with Onyx015 and Future Directions

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I. INTRODUCTION

As reviewed elsewhere in this volume, (Chap. 2), adenoviruses have proven to be one of the most promising gene delivery vectors, at least partly because of the highly efficient mechanism for cellular entry and delivery of DNA to the nucleus. Even with this most efficient of delivery systems, however, it is unrealistic to expect that biodistribution of adenovirus will be adequate to infect a high percentage of cells within a tumor. If delivered locally, physical barriers within a tumor will inevitably prevent uniform infection of the entire tumor; if delivered via the vasculature, the most efficient delivery will be to perivascular cells. One way to address the limited number of cells that can be infected has been to use adenoviral vectors containing transgenes that can kill cells that have not received the transgene, but are nearby cells that have the ''bystander effect.'' Even with genes that have a good bystander effect, the biodistribution of intravenous adenovirus primarily to liver and the reticuloendothelial system [reviewed in (1)] implies that a small fraction of virus gets to a tumor, so that dosed vector is unlikely to deliver genes to enough cells for efficacy.

An approach to address these limitations is the construction of adenoviral vectors that replicate selectively in tumors. After infection of the cells accessible to the dosed virus, the replication of the vector produces progeny virus, which then infects more cells and spreads further in subsequent rounds of infection. If the replicating and spreading virus contains a transgene, that gene is delivered to all the infected cells. This approach is quite different from the goals of most gene therapy vector designs because the cells that support replication are killed. Although that is exactly the desired endpoint in cancer therapies, it is just the opposite from the usual goal in gene therapy, which is to leave a healthy cell containing a therapeutic gene.

For replicating vectors to be a safe way to delivery genes to tumors, there must be a basis for selectivity in that replication. A fundamental insight by McCormick (2) was that adenovirus inactivates some of the same cellular pathways for its replication as are inactivated in carcinogenesis, so the viral functions that inactivate these pathways are nonessential in tumor cells where the pathways are already inactivated. This led to the development of Onyx015, the first selectively replicating adenovirus to enter clinical development. This chapter reviews the clinical development of Onyx015. Because there are other excellent reviews on this subject (3–5), this chapter focuses on more recent results and their implications for the future of selectively replicating adenoviruses.

II. ONYX015: SELECTIVITY FOR P53-DEFICIENT CELLS

The adenovirus E1B55K protein has several functions, among them the inactivation of p53. In normal cells, this is essential for efficient replication, where the early functions of adenovirus would lead to accumulation of active p53 in the absence of the E1B55K function [reviewed in (3)]. Accumulation of active p53 would lead to cell-cycle arrest and/or apoptosis, aborting the virus infection. When the E1B55K degrades p53, the virus infection can proceed and produce progeny virus. In cancer cells with p53 mutations, the degradation of p53 is not essential and E1B55K is not essential. This insight led to the first publication of a selectively replicating adenovirus, Onyx015 (2), originally called dl1520 (6).

After the original publication there was controversy about the mechanism for Onyx015 selectivity. There were examples

of cancer cells with wild-type 53, which supported replication of Onyx015, and cancer cells with mutant p53, which did not support replication. The issue of p53 positive cell lines supporting replication of Onyx015 was settled by Reis et al. (7), showing that p14arf mutant cell lines would also support replication of Onyx015. In cells with mutant p14arf, the activation of p53 in response to adenovirus infection does not occur, allowing virus replication. Because many wild-type p53 cancer cells do contain p14arf mutations, this discovery not only clarified the p53 selectivity of Onyx015, but also established a basis for broader application of Onyx015 in tumors with mutant and wild-type p53 genes.

To address the issue of p53 mutant and p14arf mutant cell lines that do not support replication of Onyx015, one must recall that E1B55K has other functions beyond inactivation of p53. It is needed for shut-off of host protein synthesis and for efficient expression of late viral genes (8). Apparently this function of E1B55K is essential in some but not all tumor cells, and some tumor cells have a function that can complement the missing functions of E1B55K. Recent results suggest that through various physical and pharmacological manipulations, it is possible to make many tumor cells that poorly support replication of Onyx015 do so efficiently (F. McCormick, personal communication, 2003), and thereby increase the utility of Onyx015 even further.

The selectivity of Onyx015 is sufficient to result in an excellent safety record after administration to over 300 cancer patients. This clinical development history is the subject of the remainder of this chapter.

III. CLINICAL DEVELOPMENT OF ONYX015: LOCAL/REGIONAL APPLICATIONS

Because of the limitations on biodistribution of adenovirus and the need for a pioneering product like Onyx015 to proceed cautiously in early clinical development, the initial clinical trials of Onyx015 were done by direct intratumoral injection. This clinical development program has been reviewed by Kirn (4,5).

The indication most extensively explored by intratumoral delivery of Onyx015 has been head and neck cancer. These studies have been published in four papers (9–12). In these studies, electron microscopic evidence was obtained for virus replication in tumor cells, and not in normal cells. Clinical activity was demonstrated by shrinkage of tumors. However, in the monotherapy trials, the responses were generally short. This led to a trial of Onyx015 in combination with 5FU/cisplatin, a standard chemotherapy for head and neck cancer (10). Preclinical data indicate that Onyx015 plus chemotherapy would have benefit over either treatment alone (13). Because this was a single-arm trial and was conducted in a patient population where some would be expected to respond to the chemotherapy alone, definitive proof of the combination activity was not obtained from this study. However, comparison with historical results, and shrinkage of virus-injected tumors but not uninjected tumors (which were also exposed to the same systemic chemotherapy) in some patients, led to the conclusion that this trial suggested activity of the combination of Onyx015 and chemotherapy.

A study was also conducted in oral leukoplakia, a premalignant condition that can progress to squamous cell carcinoma. Mutations in p53 are often detected in these lesions (in 45% of cases), which suggested that they might be susceptible to Onyx015. In this study, Onyx015 was administered as a mouthwash, in which the patients rinsed and held the virus solution for up to 30 min. In a preliminary report (14), 4 of 19 evaluable patients achieved complete histological resolution of dysplasia, and the grade of dysplasia improved in 3 additional patients. This result is encouraging that Onyx015 may have a role in treatment of premalignant lesions before progression to malignancy.

Several other studies have given Onyx015 via intratumoral injection. Pancreatic cancer has been treated by both percutaneous injection (15) and by endoscopic ultrasound guided delivery (16). Hepatobiliary cancer patients were treated with Onyx015, with report of one partial response in a patient with cholangiocarcinoma (17). An encouraging observation in the latter study was that half (8 of 16) of the treated patients had a greater than 50% reduction in tumor markers associated with a period of radiographic stable disease. Habib et al. (18) administered dl1520 via percutaneous injection to 5 hepatocellular carcinoma patients and reported 1 partial response. Onyx015 has also been delivered via intraperitoneal injection in treatment of ovarian cancer (19).

Evidence for viral replication after intratumoral administration has been obtained in various studies by electron microscopy, in situ hybridization, and circulating viral DNA. These data across the various studies have been comprehensively reviewed by Kirn (5).

IV. INTRAVASCULAR DELIVERY OF ONYX015: HEPATIC ARTERY DELIVERY

The first intravascular treatment with Onyx015 was via catheter into the hepatic artery in a phase I/II trial design. The dosing schedule was first to give Onyx015 alone in 2 doses 1 week apart. Then starting 2 weeks later, cycles of Onyx015 plus bolus 5FU/leucovorin were given every 4 weeks.

This study involved a dose escalation, starting at 2×10^8 viral particles per dose, to a top dose of 2×10^{12} particles per dose. The phase I dose escalation portion of the trial did not find a dose-limiting toxicity, and the most common adverse events reported were fever, rigors, and fatigue (20). This was followed by the phase II portion of the trial, in which 27 patients were treated at the top dose of virus on the same schedule as the phase I portion of the study (21). This expanded group of patients also demonstrated an excellent safety profile of Onyx015, with the flulike symptoms being the most common adverse events. These symptoms were consistent with the observed elevation of the proinflammatory cytokines (tumor necrosis factor-α, interferon-γ, and IL6). The occasional observations of elevated transaminases or hyperbilirub-

inemia were either judged due to disease progression, or transient and not considered dose limiting. An independent study also administered dl1520 at a dose of 6×10^{12} viral particles per dose via the hepatic artery to 7 patients, and also concluded that the virus could be safely administered by this route (22).

An important conclusion from this study was the measurement of viral DNA circulating in plasma by quantitative polymerase chain reaction. After dosing, the circulating viral DNA had a short half-life (12 min), and was reduced below or to the level of detection by 6 h. Five patients were reported to have circulating viral DNA reappear on days 4 to 5 at levels over 10-fold above the level of detection (22). This reappearance of circulating viral DNA after complete clearance of the input dose from circulation is taken as strong evidence for virus replication. Unfortunately, the quality of available biopsy material did not allow for direct microscopic evidence of virus replication in this trial.

Because this was a single-arm trial in combination with chemotherapy, there are limitations on conclusions about efficacy. However, tumor shrinkage was reported in 3 patients who were refractory to 5FU-based chemotherapy regimens (22). Furthermore, the investigators made the intriguing observation that patients receiving doses of at least 6×10^{11} particles had a median survival of 359 days, compared with 155 days for the patients receiving lower doses (23).

With the strong evidence for viral replication and suggestion of clinical efficacy, this trial provided an important observation for the development of selectively replicating adenoviruses as cancer therapeutics: virus delivered via vasculature can infect a tumor and result in productive infection and clinical activity.

V. INTRAVENOUS DELIVERY OF ONYX015

The experience with Onyx015 has provided a number of encouraging signs that this virus can be used for local-regional treatment of cancer. Although there are significant cancers that are amenable to local therapy, a large number of cancer patients need a systemic therapy that can address metastatic disease. Intravenous delivery of adenovirus results in distribution of most of the dose to the liver and spleen (1). Because Onyx015 can replicate in the tumor cells, if a small amount of virus dose reaches a tumor it should be possible to mount an infection.

The first intravenous trial of an oncolytic adenovirus was a 10-patient study conducted with Onyx015 by Nemunaitis et al. (24). This study involved dose escalation from 2×10^{10} to 2×10^{13} viral particles per dose. The virus as administered every 3 weeks, in combination with carboplatin and paclitaxel. No dose-limiting toxicity was achieved with these doses of virus, with the most common adverse events being fever, rigors, and transient transaminitis. Nemunaitis conducted treatment of an additional 10 patients with Onyx015 in combination with CPT11 and with IL2 (25). A trial of intravenous Onyx015 monotherapy was also conducted by Hamid et al. (26) in 18 patients with metastatic colorectal cancer. Habib et al. (22) reported safe treatment of 3 patients with intravenous dl1520.

In all these reports, the dosed Onyx015 was rapidly cleared from circulation. One of the patients treated by Hamid et al. (22) died from a treatment-unrelated cause 56 h after dosing, and autopsy results demonstrated that most of the viral DNA was found in the spleen and liver. However, in several of the patients in each intravenous Onyx015 study, there was viral DNA detected in plasma at 3 days after dosing at levels above what was detected after clearance. This is suggestive of viral replication. Furthermore, Nemunaitis et al. (25) obtained electron microscopic evidence of viral replication in a tumor biopsy sample.

Safety and evidence for viral replication after intravenous administration has also been reported with 1 other selectively replicating adenovirus, CG7870 (27). This virus is selective for prostate cells because of prostate-specific promoters controlling E1A and E1B.

Although there is evidence for viral DNA replication, no objective clinical response has been reported with intravenous Onyx015. Coupled with the evidence that local Onyx015 can have clinical activity, the conclusion is that, although intravenous virus does reach the tumor and replicate, there is insufficient virus and insufficient replication to produce a clinical response. An intriguing observation by Hamid et al. (26) was that the patients with plasma viral DNA evidence of replication were the ones with very low titers of adenovirus-neutralizing antibody before dosing. This suggests strategies to blunt development of neutralizing antibodies (e.g., 28) may increase the efficiency with which intravenous Onyx015 reaches the tumor on repeated dosing. Formulation of the virus to protect it from the activity of neutralizing antibodies may also address this issue (29).

VI. SELECTIVELY REPLICATING ADENOVIRUSES AS VECTORS FOR GENE THERAPY

Important future applications of Onyx015 and other selectively replicating adenoviruses are as vectors to deliver genes to tumors. The replication and spread of Onyx015 will enable delivery of an inserted gene to more cells than the initial dose reaches. An important consideration is that there would be little benefit to using a replicating adenovirus to deliver a gene that would kill or correct a defect in an infected cell because the infected cell is destined to die from the lytic infection. The goal using the virus as a vector would be to deliver a gene with a ''bystander effect'' (i.e., ability to kill surrounding uninfected cells). This may be important if there is a subpopulation or cells in the tumor that are not susceptible to virus infection.

The first clinical example of a replicating viral vector delivering a transgene was recently published by Freytag et al. (30). These investigators constructed a derivative of dl1520 in which a fusion gene of herpes simplex virus thymidine kinase (TK) and *Escherichia coli* cytosine deaminase (CD) is

expressed from the cytomegalonirus immediate early promoter, inserted into the E1B55K region of the viral genome. The TK gene product can convert ganciclovir to its toxic phosphorylated metabolite, and CD can convert 5-fluorocytosine to the cytotoxic 5-fluorouracil. The study involved dose escalation from 10^{10} to 10^{12} viral particles given by intraprostatic injection, in 16 patients with locally recurrent prostate cancer. A 1- or 2-week course of ganciclovir plus 5FC was begun 3 days after virus injection. Prostate biopsies taken 2 weeks after virus injection showed evidence of expression of the transgene in 4 of 12 patients, and evidence of necrosis of the tumors. Encouraging was a drop in serum levels of prostate-specific antigen of at least 25% in 7 of 16 patients, and of at least 50% for at least 4 weeks in 3 of the patients. Two of the patients were free of adenocarcinoma on biopsy 1 year after treatment.

A further refinement of the use of a replicating adenovirus vector comes from insertion of the transgene under late viral transcription control (31). Late genes are expressed only after DNA replication and are not expressed efficiently by Onyx015 in normal cells. This results in selective expression of an inserted transgene in cancer cells where the virus replicates. The use of replicating viruses as vectors using this technology have been reviewed by Hermiston and Kuhn (32)

VII. PROSPECTS FOR THE FUTURE

The clinical experience with Onyx015 points to several directions for its development as a local-regional therapy. Evidence for efficacy in head and neck cancer, metatstatic colorectal cancer, and oral leukoplakia support further development. Signs of activity in other diseases such as hepatocellular carcinoma and cholangiosarcoma are also encouraging. Whether Onyx015 can be used for systemic treatment of cancer depends on finding ways to enhance the activity (either delivery of a greater fraction of virus dose to the tumor, or greater replication of the virus that is delivered to the tumor) beyond what was seen in the early intravenous trials.

Use of Onyx015/dl1520 as a vector has been shown to be feasible in both preclinical (31) and clinical (30) studies. This approach may increase the anticancer efficacy of the viruses sufficiently to give systemic efficacy.

A second-generation virus, Onyx411 (33), is selective for tumors defective in the retinoblastoma pathway. This virus has a combination of features that may improve intravenous activity, including greater replication than Onyx015 in many tumor lines, and an early and very tight block in replication in normal cells. The result is that insertion of genes into this virus under late transcriptional control is even more highly selective for tumor cells than when Onyx015 is used as a vector (34; Y. Shen, unpublished data, 2003). Although showing a promising safety and efficacy profile in mice, Onyx411 or its derivatives have not yet been tested in patients.

The scope of this chapter was to review the clinical experience with Onyx015. Readers should note that there are now a variety of creative approaches for engineering adenovirus and other viruses for selective replication in tumors. The broader field of oncolytic viruses was reviewed by Chiocca (35), and some approaches of enhancing systemic delivery of adenoviruses were reviewed by Green and Seymour (1).

With the excellent prospects for Onyx015 as a cancer therapeutic and second-generation viruses that may broaden the scope of disease treatable with oncolytic viruses, it seems highly probable that selectively replicating adenoviruses will become a new method of cancer treatment. The potential for selective amplification and expression of therapeutic genes in tumor cells makes these viruses a promising platform for cancer gene therapy.

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37

Gene Therapy for the Treatment of Cancer: From Laboratory to Bedside

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I. STRATEGIES OF GENE TRANSFER FOR THE TREATMENT OF CANCER

Since the 1970s, a multidisciplinary approach combining surgery, chemotherapy, and radiation has lead to a dramatic improvement in survival for patients affected by malignant diseases. Cellular therapies, such as stem cell transplantation, have also made a significant contribution. Nonetheless, many patients are still resistant to standard therapies, which also have high and often unacceptable acute and chronic organ toxicity, with an increased risk for secondary malignancies. Therefore, new strategies are needed to improve overall survival and decrease treatment-associated morbidity.

Several approaches may be considered to incorporate gene transfer techniques in the clinical arsenal against cancer:

1. *Modification of tumor cells*, by repairing genetic defects believed to be responsible for tumoral proliferation, for example, by restoring genes controlling cellular division or that induce programmed cell death (apoptosis).
2. *Sensitization of normal tissues or tumor cells in order to modify their therapeutic index*, by introducing into the tumor cells genes encoding for an enzyme that can transform a nontoxic prodrug into an active drug, or by introducing genes into normal tissues that can protect them against the effects of antitumor toxic drugs.
3. *Modulation of tumor invasiveness*, by delivering genes that can inhibit the growth of new blood vessels to impede nutrient supply to the tumor cells (inhibition of neoangiogenesis).
4. *Enhancement of the antitumor immune response*, either by inducing the recognition of tumor cells by the host's immune system or by enhancing the cytotoxic function of immune effectors.
5. *Gene marking*. Even though gene marking is not a therapeutic intervention per se, this approach has helped investigators to understand the behavior and outcome of transduced cells once administered back to the patient. This strategy is discussed elsewhere in this book (See Chapter 44).

This chapter describes the strategies currently used in patients with cancer, with a particular emphasis on the use of gene transfer to improve the recognition of tumor cells by immune effectors.

II. MODIFICATION OF TUMOR CELLS

Progress made in the understanding of molecular aberrations has allowed a much better characterization of the different steps leading to cancer. Many of these aberrations alter key regulatory, survival, and differentiation steps of the normal cell cycle. Other aberrations lead to the production of abnormal fusion products, with subsequent gain or loss of critical functions of the cellular cycle.

In theory, gene therapy could be used to replace an inactive gene with an active one or to neutralize an abnormal function gained by a mutated gene. Inactivation of regulatory genes frequently induces tumor cell growth. For example, mutations of the p53 gene have been identified in numerous tumor models. The exact role of the wild p53 gene has not been totally identified, but its product suppresses the expression

of genes involved in cellular proliferation and may induce proapoptotic genes. Restoration of the activity of the p53 gene in p53-mutated or -deficient tumors may stop uncontrolled cell growth (1) or induce apoptosis (2). Several strategies using adenoviral (3), retroviral (4), or nonviral (5) p53 gene transfer have tested this hypothesis in preclinical tumor models (6). Clinical trials have demonstrated the potency of p53 gene transfer in hepatocellular (5), head and neck (7), and lung carcinomas (8). Objective clinical responses, including transient tumor regression, were observed in this last trial and are described elsewhere in this book (see Chapter 38). Likewise, alterations of p16 have been described in squamous head and neck carcinomas, and approaches similar to those developed for p53 mutations are underway (9).

Many other oncogenes or aberrant fusion transcripts are involved in the tumorigenic process. These oncogenes or their products are the targets of several therapeutic strategies that aim at restoring their normal function either to stop anarchic cell growth or to induce apoptosis. Oncogenes from the *ras* family (H-*ras*, N-*ras*, and K-*ras*) are activated by a simple point mutation (10). It has been possible in a lung cancer model to block the mRNA of a mutated K-ras gene and subsequently prevent the secretion of the altered protein and delay tumor cell growth in vitro and in vivo (11,12). Similar approaches have been tested to block the effects of *fos* oncogene in a murine model of mammary tumor using a retroviral vector (13). Other methods using ribozymes (14) (see Chapter 18), antisense RNA (15,16) (see Chapter 19), or intracellular antibodies (17) have shown promising results, but are too preliminary to envision clinical applications in the near future.

Numerous difficulties must be overcome before tumor correction strategies can be successful in a clinical trial. For example, first, it will be necessary to get a corrective gene into an extremely high proportion of malignant cells, although it has been suggested that there is some form of uncharacterized ''bystander'' effect on nontransduced tumor. Second, targeting metastases will usually be necessary. Third, correction of a single defect may be inadequate to actually kill the tumor cells, leaving instead a collection of ''*n*-1 cells'' (where *n* is the number of mutations required for malignancy to occur) capable of undergoing another mutation to restart the malignant process.

Hence, exploitation of the tumor correction strategy will require significant improvements in vector efficiency and targeting, and until these come to pass, the development of novel rationally targeted small molecules will likely dominate this approach.

III. SENSITIZATION OF NORMAL TISSUES OR TUMOR CELLS

A. Prodrug-metabolizing Enzyme

Introduction of a gene encoding an enzyme that metabolizes an otherwise inert molecule into a cytotoxic agent has frequently been used in tumor gene therapy. Although the herpes simplex thymidine kinase (HSV-Tk)-ganciclovir system has been most widely applied, there are more than 20 such prodrug-metabolizing enzyme (PDME) systems currently in various stages of development and/or clinical trials (18,19). For these, the concept is that the gene encoding the PDME is expressed in the cancer cell and metabolizes a small molecule to an active moiety, which then kills the tumor cell directly. The molecule may also diffuse either through intercellular gap junctions or in the extra cellular space and destroy adjacent tumor cells. In this way, transduction of even a small proportion of tumor cells can produce a large ''bystander'' effect on adjacent tumor tissue. This in turn compensates for the low efficiency of transduction achieved by currently available vectors and may help to destroy a large tumor burden.

Brain tumors were an attractive initial target for PDME gene therapy. Because the tumors seldom metastasize, the goal of the therapy is the local eradication of the tumor. Hence, the major limitation of PDME—that it requires local inoculation of a tumor with the vector encoding the gene—does not represent a major disadvantage. A number of adult studies have been performed (20) using retroviral and (more recently) adenoviral vectors (21). Twelve children with recurrent or progressive supratentorial brain tumors were enrolled, and after tumor resection, they were treated with instillation in the tumor bed of retroviral producer cells generating particles encoding HSV-Tk followed by ganciclovir administration (22). Unfortunately, disease progression was seen in 10 of 11 patients, although 1 patient remained free of progression for 18 months.

More recently, a study has been performed on patients with retinoblastoma (23,24), which is also a highly localized tumor that is conventionally treated by enucleation and/or chemoradiotherapy. Enucleation is obviously disabling and deforming, and if the tumor is bilateral it leads to blindness. The alternatives of chemotherapy and radiotherapy are less mutilating but are both associated with secondary malignancies. In a study at Baylor College of Medicine, Hurwitz and colleagues injected bilateral retinoblastomas with adenovirus (Ad) type 5 encoding the thymidine kinase gene, followed by administration of ganciclovir. Three of the first 5 patients showed substantial tumor responses with the Ad Tk and are disease free, with retained vision, at up to 1-year post therapy (25). This trial is now accruing patients with monolateral retinoblastoma.

Other suicide gene therapies are being evaluated. Among the most developed of these is the cytosine deaminase system, which converts fluorocytosine to fluorourosil (26). There are, however, concerns that this suicide system may be less potent than the Tk-ganciclovir prodrug system. Other molecules that metabolize drugs or trigger apoptotic pathways within tumor cells are also being considered. Perhaps the most important future trend is to attempt to enhance the bystander effect. At present this is mediated predominantly by transfer of the small molecule cytotoxic drug from cell to cell. However, it is apparent that at least part of the bystander effect is dependent on an immune response generated to the lysed tumor. Hence, the bystander effect in immunocompromised animals has been observed to be substantially less than in those with intact im-

mune systems. Investigators are now attempting to combine PDME genes with sequences encoding a variety of immunostimulatory molecules (see Section V.E.1), including but not limited to interleukin 2 (IL-2) IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (27–31). Data from these studies are yet to be evaluated. Efforts are also being made to generate vectors, which can selectively divide in malignant cells (conditionally replication-competent vectors; see Chapters 3 and 38) and may therefore spread their encoded PDME genes throughout the tumor bed (32).

PDME has also effectively been used as a means of controlling T cell therapies. For example, graft vs. host disease (GvHD) may occur when donor T cells are given to patients after allogeneic stem cell transplantation in an effort to treat tumor relapse (graft vs. tumor effect) or posttransplant infections. Several groups have infused donor T cells transduced with the HSV-Tk gene and reported successful abrogation of GvHD after treatment with ganciclovir (33–36). More recently, efforts have been made to induce expression of the death signal Fas in donor T cells. An inducible construct is used in which Fas expression occurs only in the presence of an orally administered small molecule (rapamycin or its analogs) that dimerises 2 individually inactive components of a Fas transcriptional regulator, leading to expression of the Fas receptor and cell death on exposure to the ligand (37).

As T cell therapies for cancer become more widespread, these suicide mechanisms will become extremely important in ensuring that the regimens are acceptably safe.

B. Cytotoxic Drug-resistance Gene Transfer

The concept of dose intensification has long been current in modern oncology, including pediatric oncology. In other words, it is believed that giving more of a cytotoxic drug over a longer period will cure a higher proportion of patients. Although there are many obvious exceptions to this rule, for many pediatric malignancies it is clear that failure to tolerate chemotherapy in the intended doses correlates well with an increased risk of relapse. For that reason, there is an interest in using genes, which will protect normal tissues while leaving malignant cells vulnerable to destruction. By increasing the therapeutic index in this way, it is hoped that more drug can be administered and a higher percentage of patients cured.

There are many different candidate drug-resistance genes to be transferred, but perhaps the most widely studied is the human multidrug resistance-1 (MDR-1) gene. The gene product acts as a drug efflux pump and prevents accumulation of toxic small molecules, including a range of cytotoxic drugs such as mitoxantrone and daunorubicin. The primary toxicity of many of these cytotoxic drugs is on hematopoietic progenitor cells. Retroviral-mediated gene transfer of drug-resistance genes into hematopoietic stem cells has until recently been difficult to accomplish. The incorporation of fibronectin together with hematopoietic growth factors into the transduction regimen, together with repeated cycles of gene transfer, has allowed a significant proportion of hematopoietic cells to be protected with expression levels adequate to reduce the sensitivity of these stem cells to chemotherapeutic agents (38). Several other drug-resistance genes are currently under study and may soon join MDR-1 in clinical trial. These include the bacterial nitroreductase gene, which protects against drugs such as thiotepa, (39) and dihydrofolate reductase mutants, which protect against methotrexate/trimetrexate (40).

There are 2 major problems with using transfer of drug-resistance genes. The lack of targeting of current vectors means that they may transduce malignant cells and normal cells, and therefore increase the resistance of both to the cytotoxic drug. Moreover, although it may be possible to protect a significant proportion of marrow stem cells, secondary toxicities to other organ systems such as skin, lung, and gut will rapidly become evident as doses are escalated because these tissues are much less readily protected than hematopoietic stem cells.

IV. ANTIANGIOGENESIS GENE THERAPY

Because angiogenesis is a prerequisite for the development of metastatic disease for solid tumors, and probably also for leukemias and lymphomas, an attack on newly formed blood vessels may help to impede the spread of disease. A number of different large and small molecule inhibitors are currently under study, and some of these are suitable for a gene therapy approach (41). For example, endostatin, a 20-kd fragment of collagen XVIII can efficiently block angiogenesis, but the recombinant protein is difficult and expensive to produce and is somewhat unstable. Delivery of endostatin in murine tumor models by several different vector systems has been able to overcome this limitation and has proved extremely promising (42–44). Similarly, angiostatin, a fragment of plasminogen, also functions as a large molecule inhibitor of vessel growth and impedes metastastic tumors. This too can be transferred (e.g., by adeno-associated virus vector) to produce benefit in animal models of malignant brain tumors (45,46).

Much remains to be learned about the most appropriate route and cell of delivery of angiogenesis inhibition, but as with any protein-based therapeutic, gene transfer should allow a continual delivery of the drug rather than the peak-and-trough concentrations that result from most forms of injection, and may thereby produce a more sustained and effective response.

V. GENE MODIFICATION OF THE IMMUNE RESPONSE

Immunotherapy represents one of the most appealing of new antitumor approaches and active immunization in particular has proved to be among the most important of these new cellular strategies because of its ability to stimulate the immune system to actively recognize and kill the malignant cells. Identification of antigens expressed on tumor cells (Table 1) and the improvements made in gene transfer techniques, together with a better understanding of the molecular and cellu-

Table 1 Tumor Antigens Recognized by T Lymphocytes

Antigen	Primary tumor[a]	Normal tissues
Differentiation antigens		
Tyrosinase	Melanoma	Melanocytes
ACE	Colon, digestive tumors	Colon
Immunoglobulin (idiotype)	B lymphomas	B lymphocytes
gp100	Melanoma	Melanocytes, retina[b]
melan A/Mart 1	Melanoma	Melanocytes, retina[b]
gp75/TRP-1	Melanoma	
PSA	Prostate	Prostate
Self-antigens expressed at low levels on normal tissues		
MAGE-1	Melanoma	Testis,[b] trophoblast
MAGE-3	Melanoma	Testis[b]
BAGE	Melanoma	Testis,[b] trophoblast
GAGE 1,2	Melanoma	Testis,[b] trophoblast
RAGE-1	Renal carcinoma	Retina[b]
NY-ESO-1	Melanoma, breast carcinoma, lung	Testis[b]
MUC-1	Breast	Lactating breast
Oncopeptides overexpressed in tumors		
HER2/neu	Breast carcinoma, ovarian carcinoma	
p53 (wild-type)	Squamous cell carcinoma	
CDK4	Melanoma	
β-catenin	Melanoma	
CASP-8	Squamous cell carcinoma	
p53 (mutated)	Many	
ras	Colon, lung, pancreas	
MUM-1	Melanoma	
bcr/abl	Chronic myeloid leukemia	

[a] Antigen expressed in more than 25% of cases.
[b] These tissues do not express MHC class-1; therefore, antigen may not be presented.

lar mechanisms involved in the immune response against cancer, have given investigators tools to manipulate the immune system to induce an efficient immune response in the tumor-bearing host.

A. Tumor Escape

Even though many in vitro cancer gene and immunotherapy studies have been published since the early 1980s, few clinical applications have been successful. Despite impressive improvements in gene transfer technology and techniques for immune manipulation, clinical efficacy is still poor. Interest is now more focused on a better understanding of the mechanisms by which tumors can escape immunosurveillance in vivo, perhaps the greatest impediment to successful application of immunotherapy strategies. Table 2 briefly summarizes the most important ways by which tumors can evade immune surveillance.

B. Antigen Presentation Defects

An appropriate pattern of T helper and T cytotoxic cells stimulation has been proved to be essential in mounting an efficient immune response. Many tumors prevent the recruitment of T cell immune response by down-regulation of major histocompatibility (MHC) molecules or of costimulatory and adhesion molecules.

Several immunotherapy strategies have been conceived to overcome these defects. Introduction of MHC molecules into tumors was one of the first strategies adopted clinically in the 1990s. Earlier murine models had shown that an increased expression of MHC class I molecules decreased tumorigenicity, due to enhanced antigen presentation to $CD8^+$ cytotoxic T lymphocytes (CTLs) (47). Tumor immunogenicity has also been increased by gene transfer of both allogeneic MHC class I and II molecules (48–50), thus demonstrating the relevance of both CD8 cytotoxic and CD4 helper T cells in enhancing

Table 2 Mechanisms by Which Tumor Can Escape Immune Surveillance

Defects in antigen presentation
- Adhesion deficiency
- MHC molecules/pathway defects
- Defects in antigen processing/transport
- Defects in costimulatory pathways
- Antigenic variants
- Decoys

Microenvironment abnormalities
- Inhibitory cytokines/ligand
- Growth/survival factors, angiogenesis
- Immune sanctuaries (eye, testis, central nervous system)
- Establishment of latency (virus-induced tumors such as EBV, HPV, and HTLV)

T cell defects
- Absence/deletion of specific T cell precursors
- Anergy
- Down-regulation of TcR ζ chain
- Mutations in signaling pathways
- Deletion/defect in helper T cell
- Defect in establishment of T cell memory
- T cell inhibitors

systemic immunity against cancer. Increased expression of MHC molecules can also be obtained indirectly by transducing tumor cells with cytokines able to induce MHC molecule up-regulation on the cell surface: examples of cytokines with this property are interferon-γ (IFN-γ) (51,52), IL-4, and Tumor Necrosis Factor-α (TNF-α) (53).

Once the T cell receptor (TcR) has specifically interacted with the epitope, costimulatory signals induce a T cell response and prevent anergy (54). A number of such signals have been identified of which the B7 family members, such as B7.1 (CD80) and B7.2 (CD86), are among the best known. These molecules are expressed on the antigen-presenting cell (APC) surface and bind to their cognate receptor, CD28, on the responding T cell (54–56). Other costimulatory molecules, such as intercellular adhesion molecules and leukocyte function-associated antigens are also important (57). The absence of costimulatory molecules on the tumor cell surface is one of the explanations for the unresponsiveness of $CD8^+$ T cells in the tumor-bearing host. This important role of CD80/86-CD28 interaction in T cell activation made B7 genes an appealing target for gene transfer into tumor cells (58–60).

CD40 ligand (CD40L) also represents a possible candidate for gene transfer because it can enhance antigen presentation by tumor cells and induce maturation of professional APCs (61,62). This molecule (normally expressed only by activated $CD4^+$ T lymphocytes) interacts with the specific receptor CD40 and increases antigen presentation by malignant cells by up-regulating costimulatory molecules (e.g., B7-1 and B7-2), adhesion molecules (e.g., ICAM-1 and ICAM-3) and MHC molecules. CD40L also increases antigen uptake by dendritic cells (DCs) and allows them to bypass $CD4^+$ T helper cells in recruiting specific CTL (63–65). Hence, CD40L gene transfer has been widely used in multiple preclinical and several clinical studies (described later in this chapter) with promising results (66–69).

Failure to take up tumor antigens or to present them adequately on APCs (mainly DCs) is commonly observed in tumors. Many immunotherapy strategies therefore aim to increase the efficiency of antigen presentation. In this context, GM-CSF has been widely used for transducing autologous or allogeneic cancer cells and proved to be one of the most potent molecules in vivo, mainly acting as a critical factor for promoting maturation of DCs at the site of vaccine injection and thereby enhancing tumor antigen uptake and presentation (70).

C. T Cell Defects

T lymphocytes in the tumor-bearing host may be decreased in number or fail to home to tumor infiltrates. They may also be anergic and show increased apoptosis, impaired cytokine secretion, inhibition of proliferation, decreased cytotoxic capacity, defects in helper function, and absence of memory T cells. There may also be an increased production of immunosuppressive factors, such as transforming growth factor-β (TGF-β) and IL-10, originating from regulatory T cells or from the tumor itself (71–73).

Multiple strategies have been used to overcome T cell defects. Most commonly, tumor cells are transduced with genes encoding cytokines able to re-create an optimal microenvironment for T cells recruitment, activation, and expansion. Several cytokines can be used alone or in various combinations to enhance antitumor T cell-mediated immunity: recruitment of cytotoxic $CD8^+$ T cells able to recognize tumor-specific antigens is observed with IL-4 (74), whereas $CD4^+$ T cell recruitment is favored by TNF (75) and IL-7 (76,77). Expression of class I antigens is enhanced by IFN-γ (78,79), which in turn increases tumor immunogenicity. A combination of these effects may also be obtained: for example, IL-2 can recruit both cytotoxic T and natural killer (NK) cells directly (80) and can also induce release of the secondary cytokine IFN-γ (81–84). NK cells may also be important in generating an effective antitumor immune response (85). These cells exert cytotoxic activity through the granzyme-perforin system and release inflammatory cytokines (including IFN-γ, IL-5, TNF-α, and GM-CSF). NK cells, unlike T lymphocytes, recognize and destroy target cells that lack MHC antigens, so they can be effective against tumors with down-regulated surface expression of these molecules. IL-2 can also induce the generation of lymphokine-activated killer cells form both T and NK cells (80,86), which have enhanced cytolytic activity against a broad range of tumor cells and act in an MHC-unrestricted manner.

Costimulatory surface molecules such as B7.1 or CD40L may serve as accessory signals in the T cell activation process and prevent/overcome the T cell anergy induced by tumors (87,88). Finally, chemokines [such as the T cell specific lymphotactin (Lpth)] (89) may be used to attract immune-effector cells to the vaccination site, thereby enhancing the probability of adequate immune activation.

D. Microenvironment Abnormalities

Tumors can secrete substances able to induce immunosuppression. The best characterized of these are TGF-β, IL-10, and vascular endothelial growth factor (VEGF). TGF-β affects CTL function, inhibiting production of Th1 cytokines (especially IL-12), down-regulating surface expression of IL-2 receptors on T cells, inhibiting antigen presentation on MHC class II molecules and decreasing surface expression of costimulatory and adhesion molecules (71–73). As described later in this chapter, T lymphocytes can be genetically modified to express a nonfunctional TGF-β receptor and overcome the inhibitory effects of this molecule. IL-10 shares many immune-inhibitory properties with TGF-β, reducing Th1 cytokine synthesis and making tumor cells insensitive to CTL-mediated lysis. IL-10 antisense gene transduction can restore immunogenicity when tumor cells produce high amounts of IL-10 (90). VEGF serves to promote tumor angiogenesis and inhibit DC differentiation. Antisense gene transduction or gene therapy with soluble inhibitory receptors represents a promising strategy to block VEGF effects (91,92).

E. Clinical Applications of Immuno-gene Therapies

Gene transfer can be used in several ways to manipulate the immune system (Table 3). Passive immunotherapy consists of the adoptive transfer either of specific antitumor immune effectors, gene modified or not, or of specific antibodies (generated ex vivo) to the cancer patient. The use of cancer vaccines in active immunotherapy aims to induce efficient and long-lasting immune responses by direct stimulation (with gene-modified cells or cell components) of the patient immune system.

1. Gene-modified Autologous and Allogeneic Tumor Cells

Several clinical applications in humans have been reported using manipulated autologous cancer cells (93). When transduced autologous tumor cell lines cannot be obtained (because the tumor is not accessible, because the tumor cells do not grow ex vivo, or because gene delivery is difficult), an immunogenic allogeneic tumor cell line can be a valid alternative. This approach unfortunately has several limitations: (1) The tumor antigens present on the autologous tumor population may be absent in the tumor cell line; (2) the antigen may be presented on a mismatched MHC molecule and, in the absence of cross-priming of host lymphocytes, may fail to be recognized by host T and cells; (3) the tumor antigen may not contain peptides capable of being presented by host APC so an immunogenic allogeneic tumor in one individual may be nonimmunogenic in a second patient with a different human leucocyte antigen (HLA) type.

Among the many immunomodulatory gene products tested to date, vaccination with irradiated cancer cells engineered to secrete GM-CSF induced the most efficient, specific, and long-lasting immunity in several murine tumor models (70). Phase I clinical trials using autologous human genitourinary cancer cells as source of cancer cell antigens have been recently concluded (94). In the first study, renal cancer cells were removed and then genetically modified to secrete high levels of GM-CSF via ex vivo transduction with a retroviral vector. Eighteen patients with advanced renal cancer were treated. Vaccine treatment triggered an anticancer immune response manifest as conversion of delayed-type hypersensitivity (DTH) skin responses against irradiated autologous cancer cells. One patient with measurable metastases treated at the highest vaccine dose level had a partial response. The safety of GM-CSF-secreting autologous cancer cell vaccines was also shown in a phase I clinical trial for prostate cancer (8 patients treated). Cells were prepared from prostate tumors by ex vivo transduction with the same retroviral vector used for the previous study. Vaccine treatment induced anticancer immunity as assessed using DTH skin testing. Serum anticancer antibodies were detected after vaccination. An autologous GM-CSF vaccine trial was also conducted in metastatic melanoma patients (95): immunization sites were intensely infiltrated with T lymphocytes, dendritic cells, macrophages, and eosinophils in all 21 evaluable patients. Metastatic lesions resected after vaccination were densely infiltrated with T lymphocytes and plasma cells, and showed extensive tumor destruction, fibrosis, and edema in 11 of 16 patients examined. Antimelanoma cytotoxic T cells and antibody responses were associated with tumor destruction. One partial, 1 mixed, and 3 minor clinical responses were observed. Results of a phase I trial in pancreatic adenocarcinoma (in which 14 patients were enrolled) have been recently published (96). The vaccine consisted of 2 different pancreatic cell lines with a *k-ras* mutation. Patients received escalating number of vaccine cells after pancreaticoduodenectomy. Twelve patients then received adjuvant radiation and chemotherapy for 6 months. One month after completing adjuvant treatment, 6 patients still in remission received up to 3 additional monthly vaccinations. Vaccination induced increased DTH responses to autologous tumor cells in 3 patients receiving the highest dose of manipulated cells. These 3 patients remained disease free at least 25 months after diagnosis.

A third approach consists of the simultaneous administration of autologous tumor cells admixed with an allogeneic bystander GM-CSF producer cell line (97). The cell line chosen (K562) lacks HLA class I or II antigens, so reduces the probability of inducing an allogeneic T cell-mediated response. In addition, the cell line produces large amounts of GM-CSF, so that few modified cells need to be given together with autologous unmodified tumor cells. This greatly increases the feasibility of the approach for clinical application.

Table 3 Strategies in Cancer Immunotherapy

Passive immunotherapy "*adoptive immunotherapy*"	Active immunotherapy "*cancer vaccines*"
Transfer of effector cells	*In vivo immunization with*
• Sensitized ex vivo	• Purified antigen
– Pulsed APCs[a]	• Immunodominant peptide
– Transduced effectors	• "Naked" DNA encoding tumor antigen
– Restricted/nonrestricted epitopes	• Recombinant viruses encoding tumor antigen
• Expanded ex vivo	• Whole tumor cells
– Bulk	– Used as APCs
– Cloned population	– Modified to attract host APCs (cross-priming)
Transfer of specific monoclonal antibodies	*Adjuvant therapies*
– Induce ADCC[b]	– IL-2, IFN-γ: systemic administration
– Coupled with toxin or radioisotope	– IL-2, GM-CSF,[c] Lptn[d]
	– IL-12: in situ administration

[a] APC, antigen-presenting cell.
[b] ADCC, antibody-dependent cell-dependent cytotoxicity.
[c] GM-CSF, granulocyte-macrophage colony-stimulating factor.
[d] Lptn, lymphotactin.

A clinical trial at Johns Hopkins University Hospital is evaluating this approach in patients with multiple myeloma.

IL-2 has also emerged as a potential immunomodulatory molecule alone or in combination with other cytokines or chemokines, such as Lptn. Clinical trials conducted by our group at St. Jude Children's Research Hospital and Baylor College of Medicine used neuroblastoma cells transduced with adenoviral vectors so that they expressed the IL-2 gene. Both autologous and allogeneic studies were instituted. In the autologous trial, patients received up to 8 injections of their own tumor cells subcutaneously. More than half the patients produced specific antibody and a specific cytotoxic T cell response directed against the autologous neuroblasts. Of 10 patients, 5 had clinical tumor responses including 1 complete and 1 very good partial response (81). In the allogeneic study, however, the immunizing cell line induced no evident specific immunity and only 1 patient showed a partial response (82). Of note, in both studies a significant number of children showed good tumor responses on subsequent treatment with low-dose oral etoposide. This interaction between genetic immunotherapy and low-dose chemotherapy has subsequently been observed in a number of tumor vaccine studies (98,99), and likely represents a genuine interactivity between these treatment modalities that may usefully be exploited for therapeutic benefit in the future.

A subsequent clinical study in neuroblastoma was based on animal data showing that the combination of Lptn, a T cell chemokine, and IL-2, the T cell growth factor, accelerated and augmented the immune response to a tumor cell line (89). Accordingly, patients received either an autologous vaccine or an allogeneic one expressing both IL-2 and Lptn (100). In the allogeneic group, it was possible for the first time to observe specific antitumor immune responses to the immunizing cell line, and 2 patients entered complete remission, which was durable in 1 (101). In the autologous study, the clinical results did not appear to be measurably superior to IL-2 alone, but a clear bias toward a Th2 MHC-restricted immune response was observed (R. Rousseau, personal communication). Hence, in the allogeneic setting at least, there is preliminary evidence that the combination of 2 agents acting at different phases of the immune response may be superior to a single agent. If these results are confirmed, they may increase the feasibility of using allogeneic vaccines with the considerable simplification in protocol development that would result.

In hematologic malignancy, a phase I study of autologous acute myeloblastic leukemia cells engineered to secrete GM-CSF has recently commenced, and this study is now being extended to pediatric acute myeloid leukemia (102). It has also proved feasible to express costimulator molecules such as CD40, CD40L, or B7.1 on primary tumor cells surfaces. We are currently using a combination of CD40L and IL2 gene transfer into pediatric acute lymphoblastic leukemia cells in an effort to generate an antitumor immune response in patients with high-risk disease who have entered remission. To date, this study has proved to be safe and has generated antileukemia humoral and cellular Th1 immune responses (103). Because these patients are treated in remission, we do not yet know whether there has been any antileukemia activity.

Genetic modification of tumor cells appears safe and is capable of generating specific humoral and cellular antitumor cytotoxic responses. There have been at least some tumor regressions and the approach is now being evaluated in a wider range of tumors and in a larger number of patients.

2. Cancer Therapy with Gene-modified T Cells

Several studies have suggested the feasibility and apparent clinical efficacy of adoptive transfer of CTLs directed at viral or tumor antigens (104–108). By using gene-marked cells in these studies, it has not only been possible to determine the survival and homing of the infused T cells, but also to determine if they mediate adverse effects such as GvHD (109,110). Clinical studies and their results are described elsewhere in this book (see Chapter 44), and include Epstein-Barr virus (EBV)-associated post transplant lymphoproliferative disorder (PTLD) (111–114), Hodgkin's disease (115), and nasopharyngeal carcinoma (116).

a. Protecting T cells Against Tumor-induced Down Regulation. Clinical studies using CTL against EBV-related malignancies are promising and there have been tumor responses, particularly in patients with PTLD. Nonetheless, no patient with aggressive relapsed EBV^+ Hodgkin's disease has been cured using EBV-specific CTL. This may be due to a lack of specificity of the EBV-specific CTL for the immunosubdominant LMP1 and LMP2 antigens that are all present on the Hodgkin tumor cells. In addition, the tumor secretes immunosuppressive cytokines and chemokines, which affect CTL function and APC activity (117).

Gene transfer can be used to overcome both types of problems. By using dendritic cells transduced with adenoviral vectors encoding either LMP2 or a mutated LMP1, it is possible to generate CTL that have high cytolytic activity in vitro to LMP2- or LMP1-positive targets when compared with conventional EBV-CTL (118–120). Although LMP2- or LMP1-specific CTL may be more effective, there is a concern that they will remain vulnerable to the immunosuppressive cytokines secreted by the Hodgkin's Reed–Sternberg cell. The cytokine that has the most devastating effects on CTL proliferation and function is TGF-β (117,121). This cytokine is secreted by a wide variety of childhood tumors and allows the tumor to escape the immune response (122). To overcome this capacity to inhibit the EBV-CTL, our group transduced CTL from patients with relapsed EBV-positive Hodgkin's disease with a retrovirus vector expressing a dominant-negative TGF-β type II receptor (DNR). This prevents formation of the functional trimeric receptor. Cytotoxicity, proliferation, and cytokine release assays showed that exogenous TGF-β had minimal inhibitory effects on DNR-transduced CTLs (123). This combination of tumor-specific and tumor-resistant CTL may prove highly effective for therapy.

b. Chimeric T Cells for Tumor Therapy. Primary T cells genetically modified to express chimeric receptors derived from antibodies and specific for tumor or viral antigens have

considerable therapeutic potential. Chimeric T cell receptors allows the recognition specificity of T lymphocytes to extend beyond classical T cell epitopes by transducing cells with genes that encode the variable domain of a tumor-specific monoclonal antibody (MAb) single-chain fragment (ScFv) joined to a cytoplasmic signaling domain. This strategy can therefore be applied to every malignancy that expresses a tumor-associated antigen for which an MAb exists (124,125). Unlike conventional T cell receptors, these chimeric receptors will be active even if the tumor cells are MHC class I negative. Chimeric receptor-transduced T cells have numerous advantages over immunotherapies based on monoclonal antibodies or T lymphocytes alone. Because there is no need to select and expand tumor-specific antigens from scanty precursors, large populations of antigen-redirected T lymphocytes can be obtained in a matter of weeks. Moreover, chimeric T cell receptors are MHC unrestricted, so that tumor escape by down-regulation of HLA class I molecules or defects in antigen processing are bypassed. Because both $CD4^+$ and $CD8^+$ T cells can express the same chimeric receptor, the full network of T cell function is directed against tumor cells. The presence of chimeric TcR-mediated effector function may be more likely to produce tumor cell lysis than humoral immune responses alone. The perforin/granzyme killing mechanism may be effective against cells that are relatively resistant to antibody and complement, whereas cytokine secretion upon T cell activation by tumor antigen recruits additional components of the immune system, amplifying the antitumor immune response. Furthermore, unlike intact antibodies, T cells can migrate through microvascular walls, and extravasate and penetrate the core of solid tumors to exert their cytolytic activity. Finally, a single T lymphocyte can sequentially kill a multiplicity of target cells.

Human T lymphocytes genetically engineered to express these recombinant receptor genes have exhibited specific lysis via the perforin/granzyme pathways, as well as cytokine secretion upon exposure to tumor cells expressing the cognate target antigen (126). Engagement of single T cell or Fc receptor chains suffices to induce cellular activation and proliferation (127–130). Adoptively transferred chimeric receptor-transduced cells were protective in murine tumor models (126,131,132). Examples of clinical applications in treatment of tumors are given in Table 4. In particular, a high proportion of neuroblastoma tumors express tumor-associated antigens such as GD_2, L1-CAM, and N-CAM. $CD8^+$ CTL clones genetically modified to express the CE7R chimeric immunoreceptor, which consists of an extracellular L1-CAM-specific single-chain antibody, transmembrane CD4, and T cell CD3 complex zeta chain, is currently being investigated in a clinical trial (133).

However, chimeric receptor signaling produces only limited activation of the T cells, and our group is currently exploring an alternative approach to increase the in vivo functionality of the cells (134,135). We have transduced EBV-specific (not primary) T cells with GD_2-specific chimeric receptor genes. In vitro we have shown that these cells can be expanded and maintained long term in the presence of EBV-infected B cells. Although they recognize EBV-infected targets through their conventional T cell receptor and thereby become activated, they are also able to recognize and lyse tumor targets through their chimeric receptors (Fig. 1). Several cycles of virus target→tumor target→virus target can be demonstrated ex vivo, implying that EBV-specific T cells expressing chimeric antitumor receptors may represent a new source of effector cells that would persist and function long term after their transfer to cancer patients (136).

F. DC Clinical Vaccines

To overcome the antigen presentation defects in antitumor immune recognition, DCs can themselves be manipulated ex vivo and used as cancer vaccines, mainly acting by priming naive T cells (137). Several methods are now available to isolate and expand DCs from peripheral blood and bone marrow, making their use feasible in clinical trials. DCs can be induced to present tumor antigens by several strategies, including feeding with tumor cell lysates and apoptotic bodies or by using tumor-derived RNA or making DC–tumor cell hybrids. Specific tumor antigen gene transfer into DC using viral or nonviral vectors is also possible (93,138).

Several clinical studies using active immunization with DCs have been completed in patients with metastatic melanoma, metastatic renal cell carcinoma, and B cell lymphoma. Most of them have used antigen-pulsed DC (93,138), but some investigators have reported induction of immune responses using gene-modified DC (139–143)

G. Nucleic Acid Vaccines

Nucleic acid vaccines induce an immune response targeted against a protein expressed in vivo subsequent to the administration of its encoding DNA or RNA. Investigators demonstrated that the intradermal and intramuscular injections of polynucleotide products generate long-lasting T cell and humoral immunity (144).

Nucleotide-based vaccines have several advantages over proteins and peptides. They provide prolonged antigen expression that can continuously stimulate the immune system, probably through an intracellular antigenic reservoir, resistant to antibody-mediated clearance. This may favor induction of immune memory even in the absence of booster immunization. Codelivery with plasmids encoding cytokines or costimulatory molecules can further enhance the immune response (145). Moreover, nucleotide vaccination leads to antigen processing through both the endogenous and exogenous pathways, so that specific CTL and helper T cells can be recruited.

In a murine model of B cell lymphoma, mice immunized with DNA constructs encoding the idiotype had specific antibody responses and were protected against tumor challenge. Use of DNA encoding a fusion protein of idiotype and GM-CSF greatly improved vaccine efficacy (146). Improvement of vaccine efficacy was also observed when the idiotypic DNA was fused to the C fragment of tetanus toxoid (147). These preliminary results indicate that DNA may be a simple and

Table 4 Examples of Different Targets of Chimeric TCR Constructed with scFvs

Target	Authors	Comment
GD_2	Rossig et al. (149)	GD_2 expressed on melanomas and neuroblastomas
GD_3	Yun et al. (150)	GD_3 expressed on melanoma cells
HMW-MAA	Abken et al. (151)	High-molecular-weight melanoma-associated antigen
Cll	Annenkov et al. (152)	Directed against type II collagen—use T cells as gene carriers in rheumatoid arthritis
EGP40	Daly et al. (153)	Colon cancer-associated antigen
EGP-2	Ren-Heidenreich et al. (125)	Derived from MAb GA733.2, which binds the epithelial glycoprotein 2 protein, which is overexpressed on a variety of human carcinomas
CD30	Hombach et al. (154)	Malignant cell population in Hodgkin's lymphoma express high amounts of this cell surface antigen
CEA	Hombach et al. (155)	Anti-CEA chimeric TCR with scFv derived from the MAb BW431/26
ErbB2	Gerstmayer et al. (156)	ErbB2 is a type I growth factor receptor overexpressed in a high percentage of human adenocarcinomas
FR	Cho et al. (157)	Folate receptor is expressed on most ovarian carcinomas and some types of brain tumors
Neu/HER2	Stancovski et al. (158)	Human adenocarcinoma-associated growth factor receptor

efficacious system of inducing immune responses against a weak tumor antigen, provided that additional stimuli are included with the DNA (e.g., GM-CSF or tetanus toxoid) (148).

H. Future Perspectives for Immunotherapy in Cancers

Tumors possess multiple and powerful strategies to escape immune surveillance and can develop new immune evasion mechanisms during disease progression. Many of these stratagems have been elucidated, and counterattack can now be planned thanks to major improvements in protein engineering, gene transfer, and cell therapy technologies. Why then have few major clinical success yet been reported? Two centuries past, in 1893, William Coley reported regression of tissue sarcoma in 10% of patients after they had been infected with live or heat-inactivated bacteria. Since then, clinical response rates after immunotherapy have not convincingly changed. Hence, our nightmare is that this is as good as it ever gets! But the reality is that, despite lack of concrete clinical progress, our increasing knowledge and skills offers a real promise of improved results in the near future. It is also clear that immunotherapy by itself is unlikely to be sufficient to eradicate tumors because human malignancies are too heterogeneous in terms of the antigens they express and their susceptibility to immune-mediate killing. Only integration with standard treatments will likely be successful. Active immunization will be used as adjuvant therapy to eradicate minimal residual disease in patients previously debulked of their tumor by surgery, chemoradiotherapy, or stem cell transplantation. Patients receiving allogeneic stem cell transplant represent an obvious paradigm. After transplantation, when a new immune system is reconstituting, cancer vaccines could induce the formation of potent and long-lasting immunity. Specific cytotoxic lymphocytes from the donor could also be expanded ex vivo in the presence of host malignant cells and administered after transplant should malignancy recur.

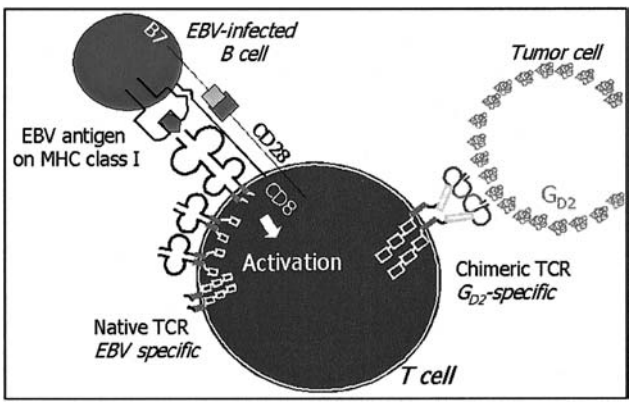

Figure 1 Chimeric anti-EBV T lymphocyte transduced with the gene encoding for the chimeric GD_2 specific-zeta chain + scFv. See the color insert for a color version of this figure.

Although no ''breakthrough'' clinical success has been reported, a better understanding of immune evasion strategies and the availability of improved technologies of immune manipulation have opened the way for real immunotherapies of cancer that should ultimately deliver on the dreams of the 19th and 20th centuries.

VI. CONCLUSION

We have far to go before gene therapy of malignancy can truly be said to have made a major impact on these diseases.

Nonetheless, over the past decade, new techniques including gene transfer and immunotherapy have produced unequivocal tumor responses even in advanced disease. Improvement of gene transfer for the treatment of cancer certainly relies on 4 major steps: (1) simplification of gene transfer protocols, still too complex to implement in a clinical environment; (2) controllable, tissue-specific regulation of transgene expression; (3) progress in the understanding of carcinogenesis mechanisms to improve therapeutic strategies; (4) improvement in the methodology of clinical trials, including optimal choice of the patient population, and monitoring of tumor and immune responses, within the tight frame of regulatory and cost-related issues. As we continue to make incremental advances in the application of these approaches, we can expect to see gene therapy increasingly supplement probably long before they can eventually supplant conventional cancer therapeutics.

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38

Gene Therapy Clinical Trials for Cancer: Replacement of Tumor Suppressor Gene p53

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I. INTRODUCTION

A. Brief History of Cancer

At one time the only treatment available for malignant tumors was surgical removal of the cancer. Surgical methods were advanced and refined in the quest to ''cure cancer,'' but radical excision alone, no matter how skillfully performed, could not provide the answer.

Armed with excised tumor tissue for study, medical science began the search for the ''magic bullet.'' Because cells proliferating out of control rapidly synthesize large amounts of DNA, radiation and chemotherapy, both inhibitors of DNA synthesis, were perfectly suited for cancer therapy. The focus was soon on finding ways to reduce the toxicity of radiation and chemotherapy by varying dosages and treatment schedules, combining chemicals, and searching for new cytotoxic chemicals. Although these tools were, and will continue to be, extraordinarily useful, DNA damaging agents alone cannot provide the ''magic bullet.''

With the discovery that the immune system recognizes tumors and often kills tumor cells naturally, the science of immunology was expected to provide ''the cure.'' Monoclonal antibody technology was a boon to cancer research, as tumor-associated proteins were zeroed in on, but hopes that these tools would provide a ''quick fix'' for cancer—that antibodies could be developed that would recognize a tumor and kill it or possibly carry toxins directly to the cell efficiently enough to cure the disease—have yet to be fully realized.

Rapid advances in genetics and molecular biology provided an even more detailed look into the machinery of the cell. Soon it became possible to identify the genes that code for proteins critical for cell function and genes that in turn regulate those genes. Once investigators were able to peer into the DNA to learn the genetic makeup of individual tumor cells, it became clear that the secret to cancer lay in the genes.

Even with the tools of molecular genetics in place, many scientists continued to denounce the theory that tumors resulted from mutations in somatic cells. Ironically, it was the discovery of a tumor virus by one scientist who initially spoke out strongly against the role of mutations in causing cancer, Peyton Rous, that eventually resulted in a universally accepted genetic paradigm that drove cancer research to where it is

today (1). Eventually, the ability to look at the genetic makeup of tumor cells provided at least one definitive answer. Cancer is caused by multiple genetic alterations in a cell that, through alterations in expression in critical genes, allow the cell to escape the natural mechanisms that prevent it from proliferating out of control.

Although highly evolved tools and techniques were necessary for cancer research to reach this point, it should not be forgotten that suspicions that we would find ourselves at this place were voiced early on. In 1914, T. Boveri suggested that a ''preponderance of the chromosomes that promote division'' might result in ''the unlimited tendency to rapid proliferation in malignant tumor cells'' (2). He continued, ''another possibility is the presence of definite chromosomes which inhibit division . . . cells of tumors with unlimited growth would arise if those 'inhibiting chromosomes' were eliminated.'' Boveri would not be at all surprised to find that replacement of tumor suppressor genes (TSG), our more elaborate term for his ''inhibiting chromosomes,'' was among the first gene therapy strategies to be successfully applied to cancer.

B. Programmed Cell Death and Cancer

An intricate web of biochemical pathways within each mammalian cell enables the cell to grow, divide, differentiate, and/or die quite independently of the organ system or of the complete organism of which it is a part. A genetically encoded developmental program, constantly tweaked by the biochemical makeup of the surrounding milleu, achieves a balance between cell proliferation and cell function consistent with the needs of the whole organism. Numerous genes are involved in an elaborate system of checks and balances that ultimately dictates, at any given time, whether a cell will proliferate or whether the cell cycle will be arrested.

During most of the lifetime of a cell, cell-cycle regulatory mechanisms restrict the cell to either G_0, where the cell is essentially at rest, or to the ''functional'' phase of the cell cycle, G_1, where genes are expressed, protein synthesis occurs, and the cell makes the proteins that are characteristic of its function. Only when appropriate is the cell released from these restrictions to progress through G_2, M, and S, the phases of the cell cycle characterized by rapid DNA synthesis and cell division. Alterations in expression of one or more critical regulatory genes, due either to inborn or acquired mutations in DNA, can upset the balance and result in blockage at a critical point in the cell cycle and/or in an inappropriate stage of differentiation.

Quite fortuitously, woven into the web of pathways is a mechanism to detect and repair defective DNA or, if repair is not possible, direct the cell to self-destruct through a process called apoptosis, or ''programmed cell death.'' Apoptosis prevents the cell from dividing and passing the genetic defect to daughter cells. Tasked with detecting damaged DNA and directing the cell to repair it or to undergo apoptosis is the TSG, p53, often referred to as ''the guardian of the genome.''

II. GENE THERAPY FOR CANCER: THE GENES AND THE DISEASES

A. Historical Perspective

The rapid development of new tools of molecular biology allowed cell biologists to examine the inner workings of cells on the DNA level and to confirm what was long suspected—that cancer is caused, in whole or in part, by genetic mutations. Following naturally from this knowledge was the desire to use the same tools to treat the disease at its core—to alter the defective DNA.

Although cancer results from genetic alterations, the array of genetic events varies widely between cancers and among patients, making it seem unlikely at first that one gene could be found that would impact a large subset of tumors. Even when a few genes were found in a large percentage of tumors, many researchers doubted that targeting a single damaged gene, when there were multiple genetic lesions present, could lead to regression of an established malignant tumor. Cancer, despite its genetic etiology, seemed an unlikely target for gene therapy.

Quite to the contrary, cancer has become the number one application of gene therapy in recent years. According to the minutes of the March 8, 2001 meeting of the Recombinant DNA Advisory Committee (RAC), 280 of the 409 clinical protocols for gene transfer therapy approved between 1989 and 2001 were for cancer.

B. The Genes

In spite of what initially appeared to be overwhelming diversity in the genetic profiles of malignant tumor cells, genetic analysis of large numbers of tumors has revealed some common patterns of mutations in 2 gene families—oncogenes (1) and tumor suppressor genes (3). Alterations in expression of genes from both families lead to disruption of normal biochemical pathways in cells—pathways governing the cell cycle, signal transduction, DNA transcription, apoptosis, cell migration, and angiogenesis—and are commonly implicated in the etiology of cancer. Although alternate pathways often exist to bypass a nonfunctional gene, the sequential acquisition of multiple random mutations eventually gives rise to cells that cannot take advantage of alternate pathways, resulting in malignant transformation.

Oncogenes are the transformed or tumorigenic versions of the proto-oncogenes that generally play prominent roles in signal transduction and transcription. Historically, these genes were relatively easy to identify for study because they stood out on genetic analysis of tumor cells as different than their normal counterparts. Tumor suppressor genes, now known to govern proliferation by regulating transcription and the cell cycle, as well as to play a major role in induction of apoptosis, were more difficult to identify as causal factors in cancer. Because the importance of TSGs to normal cell function is evident only when they are missing from the genome, more advanced gene manip-

ulation technology was required for their identification and study. The literature base on TSGs has expanded rapidly in recent years; only 10 years ago investigators, although optimistic, still spoke cautiously about how the detection of tumor suppressor genes might someday be applied to cancer. In a 1991 review article, Bishop (1) stated, ''There has been talk of restoring functional copies of tumor suppressor genes to tumors in which they are defective, but realization of this objective (if it is ever to come at all) seems many years distant.'' Instead, study of TSGs and the role their protein products play in normal cellular function has brought TSGs from the laboratory bench to the patient's bedside in a single decade.

One of the first TSGs described, *RB* (the retinoblastoma gene), has been implicated in retinoblastoma, osteosarcoma, and carcinomas of the bladder, lung, and breast. The TSG *p53* has been associated with carcinoma of the bladder, lung, and breast, along with astrocytoma and osteosarcoma. Early in the history of TSG research, particular genes were associated with Wilms tumor (*WT1*), colon carcinoma (*DCC*), and neurofibromatosis (*NF-1*). These discoveries were reviewed in depth in 1991 by Robert Weinberg (3) and by Michael Bishop (1). More recently, genes such as *PTEN* (4), *BRCA-1*, and *p16* have been recognized as TSGs, and their potential therapeutic uses are being explored.

Even with the rapid pace of developments in molecular genetics and new information about TSGs revealed daily, it is likely that we have still only scratched the surface of the base of knowledge about TSGs that will soon be available. Studies reported already though have led to the identification of genetic alterations in several TSGs, including p53, that contribute to the development of malignant tumors, and subsequently to the development and implementation of formidable therapeutic strategies.

1. Role of TSGs in Normal Cellular Function

The myriad roles TSGs play in normal cell function cannot be covered in detail in this chapter; however, a general understanding of the molecular events involved in maintaining the balance between proliferation and cell-cycle arrest is important. Briefly, expression of growth factors, oncogenes, cyclins, and cyclin-dependent kinases (CDKs) drive the cell cycle toward proliferation, while expression of TSGs and other inhibitors of CDKs induces cell-cycle arrest when appropriate.

TSGs play key roles in 2 major G_1 cell-cycle arrest pathways, the RB pathway and the P53 pathway. These tightly entwined biochemical pathways are regulated at the protein level by other TSGs and by oncogenes. In general, mammalian cell proliferation is under the control of these 2 pathways, with the Rb protein regulating maintenance of, and release from, the G_1 phase, and the p53 protein monitoring cellular stress and DNA damage, and effecting either growth arrest and repair or progression to apoptosis (5).

2. Role of TSGs in Programmed Cell Death/ Apoptosis

The importance of the tumor suppressor family of genes became evident in the early 1990s and TSGs are now acknowledged as critical elements in regulation of the cell cycle and gene transcription. One TSG, *p53*, is central to a cell's response to stress. When faced with the stress of oncogene activation, hypoxia, or DNA damage, *p53* is tasked with determining whether a cell will receive the signal to simply halt at the G1 stage of the cell cycle, will be signaled to attempt repair or, will self-destruct via apoptosis. Apoptosis plays a key role in numerous normal cellular mechanisms from embryogenesis to self-policing of DNA damage due to random mutations, ionizing radiation, and DNA damaging chemicals (6–8).

Whether a cell will be steered toward apoptosis depends on a precisely maintained balance between 2 types of signals received by a cell at any one time: proapoptotic signals vs. prosurvival signals. Although these signals determine p53's actions, expression of many of the genes that generate these critical signals is regulated by the activation status of *p53*, forming an elaborate feedback loop.

Two groups of genes are targets of p53's housekeeping duties: the ''prosurvival'' (or antiapoptotic) genes, and the ''proapoptotic'' genes. Prosurvival genes include *bcl-2*, *bcl-X2*, *bcl-w*, and *CED9*, whereas the proapoptotic genes include *bax*, *bad*, and *bid* (9). When these genes are transcribed, available transcripts of each interact with one another forming heterodimers. The relative ratio of proapoptotic to prosurvival proteins in these heterodimers determines activity of the resulting molecule, thereby determining whether the cell lives or is directed to undergo apoptosis.

3. Biochemistry of Apoptosis

Numerous genes are involved in the process of apoptosis. *Bax*, a proapoptotic gene in the *bcl-2* family, was the first gene mediator of apoptosis discovered (10). Another gene, *FAS/APO1* or the so-called ''death receptor,'' is critical for another pathway to apoptosis. Both of these pathways to apoptosis depend on p53 for induction of the caspase cascade that, in turn, is dependent on the mitochondrial enzyme cytochrome C (cytC). CytC functions as a cofactor for ATP in activation of Apaf-1, which subsequently activates caspase 9, the ''initiator'' of the caspase cascade.

Antiapoptotic (prosurvival) family members halt the caspase cascade by inhibiting the cofactor activity of cytC. For example, the p53 protein can transcriptionally induce *bax* and inhibit the activity of the prosurvival molecule bcl-2, allowing the cascade to proceed to apoptosis. Additional elegant feedback loops maintain barely detectable levels of p53 in normal, unstressed cells. Activation of p53 by stress to the cell, however, results in induction of expression of the oncogene *mdm2*. *Mdm2* encodes a protein, mdm2, which binds to and inhibits the activation of p53, closing an autoregulatory loop. Under normal, unstressed conditions, mdm2 is bound to the N-terminal transactivating domain of p53 [reviewed by (5)], prohibiting p53 activation and instead leading to rapid degradation. This precisely regulated relationship results in a very short biological half-life (20–30 min) for active p53 in the normal unstressed cell.

In the event of genotoxic stress, resulting DNA damage causes phosphorylation of serines on p53, weakening its binding to mdm2 and destabilizing the p53/mdm2 interaction. Disruption of the bond stabilizes p53, DNA-binding activity increases, and p53, through an array of downstream signals, acts to switch other genes on or off. The finely tuned sequence of biochemical events culminating in cell death requires a functional *p53* gene. Induction of apoptosis via the p53 pathway in response to random mutation, ionizing radiation, and other DNA damaging agents will not occur without *p53*.

III. REPLACEMENT OF TUMOR SUPPRESSOR GENES IN NON-SMALL CELL LUNG CANCER

A. Rationale

Targeting specific genetic lesions responsible for tumorigenesis and cancer progression is an attractive strategy for enhancing effectiveness of anticancer therapeutics as well as for reducing treatment-related toxicity. However, to be successful, a gene therapy strategy must achieve 3 goals: (1) therapeutic DNA must be successfully delivered to appropriate tissue, (2) the gene must be expressed in the appropriate target cell, and (3) expression must be subject to regulation.

Several TSGs have been successfully transferred and expressed in various tumor targets in preclinical and clinical studies. Although most cancer TSG gene therapy clinical trials to date are still in phase I, several have already provided evidence of clinical benefits to study participants and given rise to larger phase II and III trials. Several multicenter studies involving hundreds of patients are currently underway.

Some genetic alterations show up more frequently than others in tumors, but the most common damaged or missing gene discovered so far is *p53*, altered in over 50% of malignant tumors. Because inactivation of this gene through deletion or mutation correlates closely with tumorigenesis, it follows that replacement of a copy of a ''wild-type'' (wt; nonmutated) gene might restore normal regulation of proliferation. Because p53 in the normal cell is tasked with detection of damaged DNA and, when repair is not possible, induction of apoptosis, it also follows that restoring p53 has the potential to restore the normal apoptotic pathway to tumor cells. As listed in the March 8, 2001 report of the RAC, 25 protocols involving transfer of TSG DNA alone or in combination with conventional therapy were registered between 1989 and March 2001. Of these 25, 5 study protocols for *BCR-1* gene transfer in ovarian cancer were approved, along with 3 for *p16* (all prostate cancer) and 1 for transfer of the *Rb* gene. *p53* gene transfer was the most common TSG gene transfer protocol approved (16 studies), and was also being tested in the widest range of cancers: bladder (1), brain (1), breast (3), liver (1), colon (1), prostate (1), general advanced cancers (1), and lung cancer (7).

After several years of clinical trials, it now appears that the rationale for TSG gene replacement therapy in cancer is a valid concept. Because most of the TSG gene therapy clinical data to date arises from *p53* gene replacement strategies, the following discussion of the therapeutic applications of TSGs focuses on *p53*. Other TSGs, however, either alone or in combination with *p53*, also have promise as potentially beneficial therapeutic strategies.

Normally when DNA is damaged, as occurs on a regular basis by random mutation and/or by exposure to carcinogens, p53 induces G_1 arrest and directs the cell to repair the damage or to commence the pathway to apoptosis (5). Due to the critical role of p53 in the normal cell, loss of p53's cell-cycle regulatory functions, loss of the ability to self-repair damaged DNA, or unchecked expression of molecules normally regulated by the p53 protein, might make a cell more susceptible to transformation. Replacement of the *p53* gene and subsequent expression of p53 then, might be expected to restore apoptosis, increasing the sensitivity of tumor cells to conventional therapies, inducing tumor dormancy, or even preventing progression of premalignant cells to the malignant phenotype. Restoring p53 function to a cell might also suppress the tumorigenic influence of mutations in other genes critical to the functional program of the cell. Also, experimental evidence suggests a link between *p53* expression and apoptosis (11–13).

B. Preclinical Studies of p53 Gene Transfer

Preclinical studies of *p53* gene transfer into pancreatic cancer cell lines (14) and colorectal cancer cell lines (15) demonstrated suppression of cell proliferation and induction of apoptosis after transduction with *p53*. In vivo studies in a mouse xenograft tumor model also showed significant suppression of tumor growth (16). Several other lines of evidence also support the feasibility of *p53* gene replacement. For example, one study demonstrated the efficacy of *p53* adenoviral gene therapy in a mouse model of human breast cancer (17), and in another, human breast tumors were inhibited in a preclinical model of *p53* gene transfer (18).

When preclinical trials commenced, it was believed by many that the inability of a vector to transduce every cell within a tumor might limit the effectiveness of gene therapy; however, Fujiwara et al. (19) and Cusack et al. (20) demonstrated in 3-dimensional cancer cell matrices and subcutaneous xenografts that therapeutic genes were likely to spread beyond the immediate intratumoral injection site. P53 gene transfer also induced significant ''bystander'' killing in which *wt-p53* transduced cells mediated killing of surrounding cells that had not themselves been transduced possibly by mechanisms involving inhibition of angiogenesis (10,21), immune up-regulation (22–24), or secretion of soluble FAS protein (25).

Thus, despite the diversity of genetic lesions in cancer, restoration of function of a single tumor suppressor gene is sufficient to mediate tumor regression in vivo.

C. Clinical Trials of p53 Gene Transfer

In the first clinical trial of *p53* gene replacement, non-small cell lung cancer (NSCLC) patients were administered the *p53*

gene via a retroviral vector under control of the B-actin promoter (24). No vector-related toxicity was observed, and 3 of the 9 patients demonstrated evidence of antitumor activity. This was the first clinical trial to show that tumor suppressor gene replacement could mediate tumor regression in human cancer. Although this study demonstrated the feasibility and safety of gene therapy, the transduction efficiency of the retroviral vector was limiting, as is generally true of retroviral vectors. In addition, retroviruses are difficult to prepare at titers high enough to be useful in most gene therapy protocols, so subsequent trials used a more appropriate vector.

Adenoviral vectors, unlike retroviral vectors, are capable of infecting both dividing and nondividing cells and can be produced at high titers. These vectors do not integrate into the genome, however, so gene expression is transient. Although less stable expression may not be optimal in treating some diseases, transient expression is not necessarily a disadvantage in cancer therapy because prolonged expression is not required once tumor cell death has occurred.

Transfer of *p53* by an adenoviral vector Ad-p53 into lung cancer cells was demonstrated by Zhang et al. (27) and, based on these results, a phase I clinical trial with 28 NSCLC patients whose cancers had not responded to conventional treatments was initiated (28). Successful gene transfer was demonstrated in 80% of the evaluable patients, and vector-specific *p53* DNA was detected in 46%. Apoptosis was demonstrated in all but 1 of the patients expressing the gene. Most important, despite up to 6 injections per patient, there were no significant toxic effects related to transfer of the vector. In addition to demonstrating low toxicity, successful gene transfer and successful gene expression, antitumor activity was observed in 2 patients who achieved greater than a 50% reduction in tumor size. One patient remained free of tumor more than 1 year after the conclusion of therapy. Another patient experienced nearly complete regression of an upper-lobe endobronchial tumor that had resisted chemotherapy, radiotherapy, and laser treatment.

Transfer of the Adp53 construct also caused little toxicity in a phase I study of 33 patients with head and neck squamous cell carcinoma (HNSCC) (29) and, once again, significant clinical response was observed in 9 of 18 clinically evaluable patients. Interestingly, systemic Adp53 DNA was present transiently for under 48 h as demonstrated by presence in blood, urine, and sputum.

Over 200 HNSCC patients were enrolled in a phase II clinical trial of Adp53 that resulted in demonstration of complete or partial responses in approximately 10% of patients with recurrent or refractory head and neck cancer (30,31). Inclusion of those patients with prolonged inhibition of tumor growth resulted in evidence of antitumor activity in 60% patients.

Adp53's low toxicity (less than a 5% incidence of serious adverse events) suggests the potential for combining Adp53 with other anticancer treatments without significant increases in treatment-related toxicity (32).

IV. p53 GENE THERAPY IN COMBINATION WITH CONVENTIONAL DNA DAMAGING TREATMENTS

A. Rationale for Combination Therapy

Many cancer patients fail conventional therapy because their tumors are resistant to DNA damaging agents, such as chemotherapy and radiation therapy, and toxicity makes increasing doses of conventional treatments intolerable. Once apoptosis was implicated as the normal mechanism of cell destruction in response to these DNA damaging agents, it followed that a defect in the normal apoptotic pathway might confer resistance to some tumor cells. As discussed previously, p53 is the molecule that is responsible for detecting damage to DNA and directing repair or destruction through apoptosis. Interestingly, *p53* is often missing or nonfunctional in radiation- and chemotherapy-resistant tumors.

In the early 1990s, several groups (11,12,33) reported that overexpression of *p53* in cells transfected with *p53* expressing plasmids could drive cells into apoptosis or growth arrest, and also illustrated the potential for *p53* gene therapy in treating the many *p53*-deficient tumors. Subsequent preclinical studies suggested that *p53* gene replacement therapy in combination with conventional doses of radiation or chemotherapeutics may have a synergistic effect without the additional toxicity encountered with high doses of these conventional DNA damaging agents. The link between apoptosis and cell death due to DNA damaging agents was established.

B. Preclinical Studies of p53 Gene Transfer Combined with DNA Damaging Agents

Several in vitro studies (11,12,33) demonstrated that overexpression of p53 in *wt-p53* transfected cell lines could drive cells into apoptosis. Subsequent studies of apoptosis in tumor cells treated with radiation or chemotherapeutic agents, either in vitro or in animal models (4,7,34–37) also supported a link between apoptosis induction and functional p53 expression, prompting initiation of clinical trials combining Ad-p53 with the DNA damaging agent, cisplatin.

Preclinical studies of *p53* gene therapy in combination with cisplatin (38,39) showed in cultured NSCLC cells, as well as in human xenografts in nude mice, that sequential administration of cisplatin and *p53* gene therapy resulted in enhanced expression of the *p53* gene product. Pretreated cells demonstrated apoptosis in over 50% of the cells 12 h after gene transfer and in over 90% of the cells at 24 h. Cells that were not pretreated with cisplatin prior to gene transfer demonstrated only 19% and 68% apoptotic cells at 12 and 24 h, respectively. The in vivo studies demonstrated that systemic cisplatin treatment prior to *p53* gene transfer produced at least a 55% further reduction in final tumor size when compared with mice receiving gene transfer only.

Preclinical studies of *p53* gene therapy in combination with radiotherapy indicated that delivery of *p53* to *p53*-deficient

tumor cells, both in vitro and in vivo, increases their sensitivity to radiation (16). Specifically, when in vitro cultured human colorectal carcinoma cells were gamma irradiated, 55% of the tumor cells survived. Transfection of the cells with *p53* prior to irradiation, however, lowered the survival rate to 23%, and apoptosis was also increased in the pretreated cells. Furthermore, in an animal tumor model, significant tumor suppression was observed; regrowth of tumors was delayed 2 days when tumors were treated with radiation alone, and 15 days after treatment with *p53* gene transfer alone. Tumors of animals receiving the *p53* gene followed by radiation treatment, however, required 37 days to reach pretreatment size.

Other studies have generated supporting evidence for a critical link between radiation sensitivity and the ability of a cell to induce apoptosis (40–44). Data for some tumor types (i.e., epitheiod tumors) has not shown a correlation between *p53* status and radiosensitivity (45–47). However, it should be considered that when high levels of gene expression are forced with vector transduction the result may be an altered cell state quite different from cancer cells, which retain low levels of wt *p53* expression or have circumvented *p53*-mediated apoptosis during malignant progression.

C. Clinical Trials of Combination Therapy: p53 and Cisplatin

A phase I trial of *p53* in sequence with cisplatin carried out by Nemunaitis and coworkers (50) enrolled 24 patients with NSCLC previously unresponsive to conventional treatments. Patients received up to 6 monthly courses of intravenous cisplatin, each followed 3 days later with intratumoral injection of *p53*. Seventeen patients remained stable for at least 2 months, 2 achieved partial responses, and 4 continued to exhibit progressive disease. One patient was unevaluable due to progressive disease. Analysis of apoptotic activity in tumor biopsies resulted in 14% indicating no change, 7% demonstrating a decrease in apoptosis, and 79% showing an increase in number of apoptotic cells. Notably, 75% of the patients entered into the trial had previously demonstrated tumor progression on cisplatin- or carboplatin-containing regimens.

A phase II study in metastatic NSCLC evaluating patients with 2 comparable lesions was carried out by Schuler and colleagues. All patients received chemotherapy, either 3 cycles of carboplatin (AUC 6) plus paclitaxel (175 mg/m^2), or cisplatin (100 mg/m^2) plus vinorelbine (25 mg/m^2). In addition, Ad-p53 was injected directly into 1 lesion, whereas the other lesion was used as a control and was not injected. The authors hoped to demonstrate enhanced radiological response in the injected lesion compared with the noninjected lesion (49). As was observed with the Nemunaitis study (48), Ad-p53 (SCH 58500) resulted in minimal vector-related toxicity and no increase in chemotherapy-related adverse events. Although the authors concluded that Ad-p53 did not provide additional benefit to patients receiving an effective first-line chemotherapy, mean local tumor regression as measured by size was significantly greater in the Ad-p53-injected lesion compared with the control lesion with the less successful cisplatin and vinorelbine regimen. The implications of this trial are that the combination of Ad-p53 and chemotherapy agents does not significantly increase overall toxicity and confirms the observation that Ad-p53 can safely be delivered with platinum-based chemotherapy regimens.

A phase I/II clinical study of recurrent ovarian cancer patients by Buller and coworkers demonstrated safety and tolerability of single-dose and multiple-dose intraperitoneal Ad-p53 in combination with platinum-based chemotherapy (51). A long-term follow-up study (52) of these patients resulted in median survival of 12 to 13 months for individuals who received multiple-dose Ad-p53 with chemotherapy, compared with only 5 months for those treated with a single dose of Ad-p53. There were 10 long-term survivors more than 20 months after multiple-dose treatment for recurrent disease compared with only 2 long-term survivors after a single dose of Ad-p53. The authors concluded that the 12- to 13-month median survival in a heavily pretreated population with recurrent ovarian cancer compares favorably with the 16-month median survival for individuals treated with paclitaxel at the time of initial recurrence of this disease and is more than double the 5-month survival seen with palliative radiotherapy or paclitaxel failure.

D. Clinical Trials of Combination Therapy: p53 and Radiation

Preclinical studies also suggested that *p53* gene replacement might confer radiation sensitivity to some tumors (16,40,42–44) leading to the initiation of a phase II clinical trial of adenoviral-mediated *p53* gene transfer in conjunction with radiation therapy. Preliminary data from 19 patients with localized NSCLC (54) revealed a complete response in 1 patient (5%), partial response in 11 patients (58%), stable disease in 3 patients (16%), and progressive disease in 2 patients (11%). Two patients (11%) were nonevaluable due to tumor progression or early death. Examination of biopsies taken 3 months following completion of therapy revealed no viable tumor in 12 patients (63%) and viable tumor in 3 (16%). Tumors of 4 patients (21%) were not biopsied because of tumor progression, early death, or weakness. These results compared favorably with the 17% reported in studies of chemotherapy combined with radiation therapy (55).

The encouraging results of these studies of combination therapy are the basis for a currently ongoing multicenter randomized clinical trial in patients with unresectable NSCLC. This study compares a conventional therapeutic strategy (concurrent radiation and chemotherapy) with concurrent chemotherapy and radiation in conjunction with intratumoral injections of adenoviral *p53*.

V. FUTURE DIRECTIONS IN TSG GENE THERAPY

In spite of the promise apparent in these early trials, tumor suppressor gene therapy of cancer is not widely applicable in

its current form. Improvement in gene delivery systems is a critical area for future development, as systemic administration of *p53* cannot be considered using current vector systems. In addition, strategies can be developed to take advantage of bystander effects, and strategies combining TSG therapy with transfer of genes aimed at blocking angiogenesis or enhancing the immune system are also on the horizon. Ongoing exploration of TSG replacement as an adjuvant with conventional chemotherapy, radiation therapy, and surgery continues to look positive.

A. Vector Development

To date, promising approaches to gene therapy in clinical studies have used retroviral, adenoviral, and herpes vectors for gene delivery. However, due to various complications caused by injecting viral material with the genetic material, new modes of delivery using nonviral vectors and naked DNA are being explored.

Liposomes as gene delivery vehicles have shown promise for potential systemic delivery of genes to distant sites with minimal toxicity. Ramesh and coworkers demonstrated liposome-mediated gene delivery in animal studies using extruded DOTAP:Chol liposomes. Transgene expression was observed in 25% of the cells in each tumor, and significant suppression was seen in both primary and metastatic lung disease. Repeated treatments resulted in a 2.5-fold increase in gene expression and an increase in therapeutic efficacy compared with single treatments (56).

B. Tissue Targeting

A current limitation to gene therapy is the nonspecificity inherent in available gene delivery methods. So far, only tumors accessible by needle or by endoscopy are viable targets for gene therapy—tumors that are unreachable or undetectable are excluded by current gene therapy delivery methods. Thus far, recombinant adenovirus has proven the most efficient at systemic delivery of genes to less accessible tissues, such as heart and skeletal muscle. Several strategies designed to target recombinant adenoviral vectors to specific types of cells by manipulating cell surface binding properties of the viral particle have been studied.

One targeting strategy under development involves bifunctional fusion proteins consisting of an antibody fragment specific to the fiber protein of the virus and a ligand, in this case, epidermal growth factor (57). The bifunctional fusion proteins blocked normal fiber binding to the coxsackie-adenovirus receptor, with concomitant targeting of the virus to the epidermal growth factor (EGF) receptor. Addition of the fusion proteins to the adenovirus then enhanced the transduction efficiency of the epidermoid carcinoma cell line A431 16-fold at optimal conditions compared with infection with native adenovirus vector. The results of this study demonstrated blocking of the native viral tropism as well as targeting gene delivery specifically to the EGF receptor by the modified adenovirus.

C. TSG Replacement Combined with Other Gene Therapy Strategies

Although the mechanisms that *p53* and p16INK4 use to cooperate in apoptosis induction are not yet fully understood, recent reports indicated that complementary tumor suppressor genes, delivered together, might cooperate to induce apoptosis. Combinatorial introduction of the tumor suppressor gene *p16INK4* and *wt-p53* demonstrated a synergistic effect on the induction of apoptosis in HuH7 hepatocellular carcinoma cells (mutated *p53*) and LOVO colon carcinoma cells (very low expression of *wt-p53*) in vitro.

VI. FROM CLINICAL TRIALS TO STANDARD OF CARE: GENE REPLACEMENT THERAPY AND SOCIETY

Gene therapy technologies are rapidly becoming a reality with the potential to impact the general population in the forseeable future. It is, therefore, a critical time for researchers, clinicians, patients, and the public to address societal issues that could bring application of gene therapy to a grinding halt.

The first human gene transfer protocol was approved in 1988, and the number of protocols approved in subsequent years has increased dramatically—from 2 in 1990, to 43 in 1995, and then to 99 in 1999, with 70 more added in 2000. Although a majority of the trials are still at the stage of assessing toxicology and pharmacology, the number of potential applications of recombinant DNA technology to clinical medicine can only be expected to expand as successful outcomes are reported.

The transfer of recombinant DNA to humans in the last decade has raised new and complex scientific, medical, ethical, and social issues that justify enhanced oversight by regulatory agencies. Because of enhanced real and theoretical risks, gene transfer clinical trials are subject to more regulatory and advisory oversight than many experienced clinical investigators may be used to encountering. This should not be perceived as an impediment to the progress of gene therapy research, but as a method of ensuring rapid progress in the development of gene therapy strategies that reflect safe, ethical, scientifically sound, and socially responsible medicine. If gene therapy is to take its place as the standard of care for gene-based diseases as quickly as possible, clinical trials must be conducted with utmost care and integrity.

A. Public Perception of Gene Therapy

The most flawless gene therapy technologies will be of little use to a public who does not understand, and is therefore not willing to accept, its benefits. After science and medicine develop and test the tools of gene therapy, the future of gene therapy still depends on the acceptance of this new and sometimes intimidating technology by the public. Many consumers are currently wary of any type of gene manipulation, and this

aura of discomfort is likely to linger until medical science can assure them that responsible application of gene technology is a far more likely scenario than one in which the technology is allowed to extend to unethical applications. In spite of the obvious promise evident in the results of the clinical trials of TSG gene transfer described in the previous section, it is critical to recognize that there are still gaps in knowledge and technology that must be filled before the most finely tuned gene therapy strategies can emerge

The most efficient method of successfully delivering gene therapy to health care consumers will be to gain public acceptance of the technology through education in basic molecular genetics and its applications, through release of accurate information (both positive and negative) from clinical trials, and through honest discussion of the limitations, as well as the promises, of gene therapy. An official step in this direction was taken in 1974, when the RAC was formed in answer to public concerns about the safety of gene manipulation

B. Educating the Public

The day that a patient is diagnosed with terminal cancer is not the time to teach scientific literacy to the patient or his or her family members. Now is a critical time to educate the public about general genetics, and about the potential of the field of gene therapy to improve length and quality of life for all health care consumers. Only then will patients be capable of making informed decisions at diagnosis. Outreach projects such as the multimedia educational kit, ''The Human Genome Project: Exploring our Molecular Selves,'' produced in 2001 by the National Institutes of Health (NIH) and numerous cosponsors and distributed free of charge on the Internet will have an enormous impact on the public perception of the potential usefulness of gene therapy. Only education of individuals will facilitate the exchange of ideas between the public, the medical community, and governing bodies as they work together to decide how to best use this new technology in an ethical, responsible, and fair manner.

Electronic access to information on cancer clinical trials is now widely available to private physicians and potential patients through various websites. In addition, the NIH Guidelines for Research Involving Recombinant DNA Molecules: RAC Guidelines (revised January 2001) prepared by the RAC can be accessed online by physicians, scientists, patients, and the public. The RAC database listing all approved clinical trials, as well as minutes of their review meetings, are available on the NIH Office of Biomedical Affairs (OBA)/RAC website. In addition, reports of adverse effects reported to the OBA are now accessible to other investigators and the public under the Reports section of the RAC website. In addition, most cancer centers with ongoing clinical trials provide educational materials to patients and physicians.

VII. SUMMARY

Although limitations still exist to the widespread application of gene therapy to cancer, the strategy has already been shown to be applicable in several clinical situations. Contrary to initial predictions, virus-assisted gene transfer has been shown to be even more efficient in cancer cells than in normal tissue cells, and viral vectors appear to spread readily through a tumor and to cause cell death via apoptosis. Initial concerns that the diversity of genetic lesions in cancer cells would prevent the application of gene therapy to cancer appear unfounded; on the contrary, correction of a single genetic lesion has, repeatedly, yielded significant tumor regression, and successful early clinical trials of *p53* gene replacement have provided information that will be useful in the design of future gene therapy strategies.

Development of more efficient vectors, novel genes, and combined modality approaches are on the horizon and will widen the applicability of gene therapy to systemic administration. Many studies suggest great potential for combining gene therapy and pharmaceutical, immunological, and radiotherapeutic approaches to kill cells more effectively and in greater numbers. Because gene therapy targets the etiology of the disease, these strategies may someday even be applied to cancer prevention.

Unresectable tumors are a prominent problem in oncology, with proven therapies such as radiotherapy and chemotherapy controlling less than 50% of lung cancers. Clinical trials of *p53* gene replacement have provided information useful in the design of future gene therapy strategies. Direct intratumor injection has low toxicity and thus can be readily combined with existing treatments. Postinjection gene expression can be documented and occurs in the presence of an antiadenovirus immune response. Importantly, this treatment can cause tumor regression or prolonged stabilization. Novel vectors will likely allow the application of this strategy to systemic cancer treatment.

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39

Ex Vivo Gene Therapy for Alzheimer's Disease

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I. INTRODUCTION

Trophic support is essential for the normal function of the central nervous system (CNS). In addition to supporting neuronal growth, migration, and targeting during development, trophic factors appear necessary for ongoing neuronal function, cell repair, and survival. Lacking such support, the dependent cell populations may lose their functional connections, atrophy, and die. Thus, deficient trophic function could form the basis for the genesis of some neurodegenerative diseases. In addition, even in cases in which trophic loss is not the inciting event, providing ongoing trophic support may prevent the cell loss that generates a clinical disease.

II. PATHOPHYSIOLOGY OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the prototypical adult neurodegenerative disease. It is the most common cause of dementia in the elderly. With advancing age, AD becomes epidemic: it doubles in prevalence every 5 years after age 60, so that by age 95, 48% of individuals are affected (1). With lifespan lengthening in many societies in the world, an increasing number of individuals will live to confront the stark reality of this disease in themselves or someone they know (2,3).

AD occurs in both sporadic and inherited forms (4). A similar disease also appears in individuals with Down syndrome (5). Clinically, AD manifests as a progressive dementia, affecting higher-order cognitive functions such as memory, attention, language, judgment, and spatial reasoning. Patients often experience depression, irritability, aggression, and delusions. With time, the disease progresses to severe impairment of cognitive function, with preservation of the primary motor and sensory pathways. Gross pathological inspection of the AD brain reveals widespread loss of brain volume. Decreased gyral bulk and commensurate sulcal widening are evident. At the microscopic level, loss of synaptic connections between cortical neurons and the subcortical cholinergic system begins early and becomes profound as the disease progresses. The cortical neurons themselves are not affected until late in the course. The pathological signature findings are neuritic plaques and neurofibrillary tangles (6). Neuritic plaques contain insoluble β-amyloid, which is known to be neurotoxic. Neurofibrillary tangles contain intermediate filaments of α-synuclein, and likely represent ''ghosts'' of degenerated neurons. A chronic inflammatory reaction occurs in AD, with infiltration of microglia and macrophages in the region of senile plaques. Regional immunohistochemical analysis of specific cell populations in AD demonstrates cell loss in a host of different cell populations. Among these diseased cell populations, loss of cholinergic activity is prominent (7). In particular, cell loss occurs in the neurons located in the basal forebrain nuclei. The degree of cholinergic cell loss correlates well with the clinical disease (8).

The underlying etiology of AD remains ill defined. One hypothesis posits that amyloid plays the major role in the disease. In familial cases, a genetic defect in the amyloid precursor protein (APP)-processing enzymes presenilin-1 and presenilin-2, or APP itself, leads to amyloid accumulation and subsequent clinical AD (9). However, a pathologically identical disease occurs in the 90% of cases that are nonfamilial without a consistent identifiable genetic defect.

III. CONVENTIONAL TREATMENTS OF AD

Each perturbation observed in AD has been considered for potential therapeutic manipulation. AD involves a combina-

tion of deranged amyloid processing and metabolism, neurotoxicity, inflammatory response, loss of synaptic connections (especially cholinergic), and secondary cell senescence and death. Accumulation of amyloid and defects in amyloid processing has proven difficult as a target for therapy. The inflammatory reaction seen in the brain and oxidative stress have led to the long-term use of nonsteroidal anti-inflammatory drugs, which have been shown in some studies to decrease the risk of AD (10). Alpha-tocopherol (vitamin E) and ascorbic acid (vitamin C) may slow the progress of the disease (11). The loss of cholinergic activity correlates well with the magnitude of the disease, but medical therapy with acetylcholine, acetylcholine precursors, or cholinergic agonists has proven impractical due to the widespread nature of the cholinergic system throughout the body and the relative impermeability of the blood–brain barrier. Cholinesterase inhibitors, which work by preventing the breakdown of acetylcholine in the synapse, have achieved moderate success in clinical trials (12). Most oral pharmacological therapy for AD uses this strategy. Examples of such medications in clinical use include tacrine, donepezil, rivastigmine, and galantamine (13). On the whole, all cholinergic medications have had only limited success.

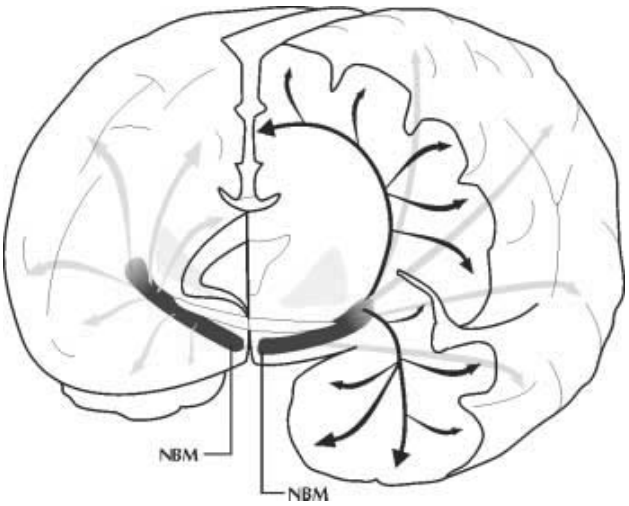

Figure 1 The diffuse connections between the NBM and the cortex are illustrated. See the color insert for a color version of this figure.

A. Trophic Factor Gene Therapy: Rationale

Optimal therapy to treat AD would be correction of the underlying defect(s) that produces the cascade of events culminating in the clinical disease. Dysfunction and death of the cholinergic cells of the basal forebrain nuclei correlate best with the clinical severity of AD. This makes therapy directed to correcting cholinergic dysfunction desirable. However, cholinergic supplementation therapies have met with limited success. An attractive approach would be the preservation and/or augmentation of the cholinergic system.

1. The Cholinergic System in AD

The classic pathological hallmarks of AD are the formation of neurofibrillary tangles and amyloid-containing neuritic plaques. These changes can occur without clinical dementia, and they are not good independent predictors of the severity of dementia. Although cell loss in AD eventually becomes widespread and involves multiple neurotransmitter populations, cholinergic cell death in particular correlates with the clinical disease and degree of synaptic loss (14). The basal forebrain nuclei are the most prominently affected. These include the nucleus basalis of Meynert (NBM), the medial septal nuclei, and the vertical limb of the diagonal band. The connections of these nuclei with the cortex are widespread (Fig. 1). Evidence of the importance of the cholinergic system in AD is supported by the observation that cholinergic antagonists can reproduce or exacerbate the symptoms of the disease, while cholinergic agonists improve the symptoms of AD (15). Clinical and experimental lesions in the cholinergic system disrupt attention and spatial memory, both of which are prominent features of AD (16–18). Importantly, damage must be widespread throughout the cholinergic system in animal models to produce such symptoms as memory deficit, suggesting that preservation of even part of the system might be enough to prevent clinical impairment (19). A number of animal models of aspects of AD exist, including injection of ibotenic acid or other toxins into the septal region and section of the fimbria/fornix. Fimbria/fornix lesions in particular disrupt the reciprocal connections between the basal forebrain cholinergic system and the hippocampus leading to substantial loss of cholinergic neurons.

a. Nerve Growth Factor. Nerve growth factor (NGF) is the prototypical member of the neurotropin family. It is a low-molecular-weight polypeptide with trophic effects for cholinergic neurons. NGF up-regulates choline acetyltransferase. The role of NGF in the support of the cholinergic system was elucidated through a series of studies that first examined the anatomical distribution of neurons that expressed NGF and NGF receptors. Later studies examined the effect of NGF on normal and injured target cells. Medial septal cholinergic neurons express both low-affinity (p75) and high-affinity (*trkA*) NGF receptors on their cell bodies and their terminals, which project to the hippocampus (20–22). The NGF that is expressed by cortical neurons binds to these receptors on cholinergic fibers and is transported retrograde to the cell bodies in the NBM (23). Fimbria/fornix lesions in both rats and monkeys that interrupt this pathway lead to the degeneration of cholinergic neurons (24,25). NGF infusion into the cerebrospinal fluid of lesioned macaques specifically prevented cholinergic neuron degeneration in the basal forebrain nuclei (26,27). NGF infusion studies in rats and subsequently in nonhuman primates showed that NGF infusion could ameliorate or even reverse age-related pathological changes. In the rat, this is associated with improved performance on tasks of spa-

tial memory (28). An alternative method of NGF delivery is the in vivo gene transfer of NGF using an adeno-associated virus (AAV) vector injected directly into the medial septal nuclei (29). This treatment reduced cell loss following a subsequent fimbria-fornix lesion when compared with placebo. Long-term transduction and cell survival (at least 6 months) of medial septal neurons transfected with AAV carrying either hBDNF or NGF is also observed (30). Last, grafting of fibroblasts modified to secrete NGF into aged rats demonstrated survival of the grafted cells, an increase in basal forebrain low-affinity NGF receptors (p75), and improvement on memory tasks (31). Neuronal rescue has also been observed in primates receiving grafts of autologous fibroblasts genetically engineered to secrete NGF (32). These studies illustrate the importance of NGF in basal forebrain cholinergic survival and function.

In addition to NGF, another trophic factor, glial cell line-derived neurotrophic factor (GDNF), also prevents cell degeneration in rats with fimbria/fornix lesions. However, it is 10 times less potent than NGF (33).

b. Trial of NGF Infusion in AD. Theoretically, infusion of a trophic factor into the cerebrospinal fluid (CSF) in the proximity of a target would appear to be a viable delivery method. This formed the basis for a clinical trial of intraventricular infusion of NGF in patients with AD (34). However, the development of wasting and painful syndromes led to cessation of the trial. The genesis of these unpleasant side effects appeared to have resulted from the circulation and action of NGF on other responsive brain targets bathed by the CSF. Animals so treated have been shown to stimulate schwann cell proliferation and migration, as well as sprouting of sensory and sympathetic neurons (35,36). These effects appear reversible with cessation of the infusion. Clinically, weight loss in animals, believed to be due to the action of NGF on the nucleus accumbens, and a pain syndrome in 3 human patients have also been seen (37–40). These observations suggest that targeted delivery of NGF for trophic factor therapy is essential.

B. Gene Therapy for AD: Gene Delivery

There are 3 major issues in the introduction of DNA for large molecules such as trophic factors to the brain. First, the CNS exists in a privileged environment that is sheltered from the rest of the body, including the immune system, by the blood–brain barrier (BBB). Second, most neurons in the adult CNS are postmitotic and therefore do not easily incorporate new DNA once introduced. Third, trophic factors need to be selectively targeted to the intended anatomical region or cell population. The BBB excludes most gene therapy vectors. Naked DNA, most viruses, and all but a few cells will not cross into the brain unassisted. Viruses that can penetrate the BBB and transduce CNS cells include herpes viruses, retroviruses, adenoviruses, and AAVs. However, these viruses lack the transduction efficiency and target selectivity for individual cell populations necessary to make them useful in the treatment of AD.

In sum, the nonselective infusion of trophic factors or their expression constructs does not offer the necessary specificity of targeting for selective therapy because the introduced factor can be distributed to other active sites to produce untoward side effects. In contrast, ex vivo gene therapy, in which cells are modified in vitro and subsequently placed precisely into their targets using stereotaxic surgery, circumvents the BBB, removes the concern about transduction efficiency, and allows for precise spatial selectivity.

C. Ex Vivo Gene Therapy: Rationale and Considerations

Ex vivo gene therapy is one form of cell-based therapy. It is similar to other cell-based treatment modalities in that all require the selection of a cell population that is immunologically compatible, nonproliferating, and robust enough to survive after implantation into specific target sites and also produce the desired effect(s) for the lifetime of the patient. Cell-based treatment strategies that have been explored for the treatment of neurodegenerative disease include (1) the supplementation of neurotransmitters that are deficient due to cell loss through the implantation of cells exhibiting the same neurotransmitter phenotype; (2) the implantation of fetal brain cells or stem cells that potentially could differentiate into region-specific cells to replace lost neurons; and (3) the prevention of progressive cell loss by providing trophic factor(s) essential to cell survival and function. The first attempt at cell implantation therapy into the CNS for neurodegenerative disease involved the grafting of a patient's own adrenal medullary cells into the caudate for the treatment of Parkinson's disease. Early enthusiastic results did not survive more rigorous scrutiny. However, this initial failure did little to dampen hopes that one day neurodegenerative diseases would be treated successfully by grafting. This approach, which is essentially a neurotransmitter replacement strategy, is inadequate for AD because of the diffuse nature of the functional connections that need to be sustained; simply applying more transmitter does not replace the lost function. Where it has been used in other diseases, supplementation therapy using neurotransmitter agonists has only been successful in addressing selective symptoms. Other strategies ultimately must address the pathophysiology more directly.

Grafting fetal human or animal brain cells represents a logical next step. This strategy has been tried both experimentally and clinically. Investigators' initial hope was that fetal cells, given their pluripotent properties, would have the ability to adapt to their new environment and automatically replace or restore failing cells. This has proved somewhat vain, but the strategy continues to hold some promise; experimental efforts to use grafted fetal/progenitor cells in this fashion continue.

The use of ex vivo modified cells to deliver trophic factors is a potentially promising cell-based strategy for the treatment of AD. In this strategy, cells in an established culture are transduced to produce a trophic factor and are then implanted at the desired site of action. Because NGF secreted by geneti-

cally engineered cells does not diffuse through the brain far from its point of release, collateral effects should be limited. Although this is an advantage from the standpoint of side effects, it comes at the cost of the risk and trouble of surgically implanting the cells precisely at every target that needs trophic support.

1. Cell Selection for Gene Therapy Grafting

Various cell types have been used as carriers for the introduction of selective genes into the brain. These include stem cells, neural progenitor cells (NPCs), and fibroblasts. Each type of cell has different advantages and liabilities in terms of host compatibility, functionality, and oncogenicity.

Stem cell lines of different ages (e.g., embryonic and fetal) and different origins (e.g., brain and blood) have been established and characterized for use in cell replacement therapy. Embryonic stem (ES) cells can potentially provide a rich source of undifferentiated cells from which cells of specific functions can be derived. These differentiated cells can then replace or augment specific cell populations in the diseased brain. The desired cells are generated from the undifferentiated cells by providing them with a series of staged signals. Dopaminergic neurons have been generated from a population of ES cells using a plasmid expressing nuclear receptor related-1 (Nurr-1) transcription factor and a 5-stage differentiation method (41). Implantation of these altered cells corrected certain neurological deficits in one model of Parkinson's disease. Such a strategy may be useful in future cellular therapy for Parkinson's disease. A similar strategy that generates NGF-secreting cells similar to those that normally provide trophic support to the basal forebrain nuclei could provide a cell source for implantation.

Immortalized NPCs represent another renewable population of cells that can be transduced ex vivo to secrete NGF. An advantage of using these cells is that large numbers can be grown and prepared, and they should adapt well to the CNS environment. In one study, NGF-secreting NPCs were implanted into the medial septum in aged, previously unoperated rats. Animals that received grafts did not undergo the age-related decline in performance on a Morris Water Maze spatial learning task that the control animals did (42). In another study, rats that received NPC grafts expressing NGF factor showed increased hippocampal CA3 cell survival and neuromotor function following experimental head trauma (43). Graft survival and cholinergic sprouting in the implantation bed have also been observed after placement of NPCs transduced with human brain-derived neurotrophic factor (hBDNF) into adult rats (44).

Last, transduced skin fibroblasts have been used as vehicles to introduce specific genes into the CNS. In this method, skin biopsies are performed and the fibroblasts are cultured. A variety of methods for transducing the fibroblasts are available. The gene of interest is encased in an appropriate vector. Possible vectors include retrovirus, lentivirus, adenovirus, AAV, or DNA constructs such as plasmid DNA or lipsome-encased DNA. The vector is introduced into the culture. The transduced cells are then isolated and expanded. The viability and reliability of transduced fibroblasts as vehicles for delivering NGF to the CNS has been demonstrated in a number of studies (45,46). Genetically modified fibroblasts grafted into the spinal cord have been shown to survive, produce NGF, and promote the growth of specific neuronal subtypes (47). In transplanted tissue, the NGF produced is released intraparenchymally and has a restricted area of action.

2. Considerations for the Application of Ex Vivo Gene Therapy to AD

Rodent and primate studies in cholinergic denervation indicate that NGF is important in rescuing cells from axotomy-induced cell death as well as potentially augmenting cholinergic function. Therefore, to evaluate the effectiveness of NGF as a therapy for AD, patients to be treated would ideally be in the early stages of the disease, so that the rate of disease progression can be assessed. Outcomes should be assessed with careful preoperative and postoperative monitoring with standard psychological batteries, AD and other dementia rating scales, and magnetic resonance imaging (MRI) of the brain.

To implement ex vivo gene therapy for AD, a population of cells needs to be genetically modified to secrete NGF. For reasons relating to their immunology, autografts of patient's skin fibroblasts appear to be the most promising vector. Animal experiments have confirmed the practicability of this strategy. The transduction of these cells can be effected by the culturing of cells harvested from patient's own skin, genetically modifying them with the application of retroviral constructs, and expanding and selecting the cell population of interest (Fig. 2). The level of trophic factor production per cell can be determined. These cells are then concentrated into a small volume for injection in a dose-specific manner.

Next, the genetically modified cells must be implanted into the NBM. To successfully introduce the cells, the exact location of the NBM must be determined and readily confirmed using radiological scanning techniques. The nucleus is situated between the anterior commissure and the base of the brain. An appreciation of its location and morphology can be achieved by examining immunostained brain sections. This anatomic knowledge can then be correlated with MRI of the appropriate sections of the brain in individual patients (Fig. 3). This then permits the determination of the coordinates of each target in the basal nucleus. The major consideration in the design of the operative procedure is the need for precise targeting of the implants while minimizing the risks of implantation. Any implantation procedure carries with it, by its necessarily invasive nature, risks of hemorrhage and infection. Stereotaxic implantation of these cells affords the most precise targeting. This involves placement of a stereotactic head frame, imaging of the brain while in the frame, determination of the coordinates for each specific target, placement of a bur hole or bur holes, and introduction of an injection catheter under precise control. Accurate graft implantation can be complicated by the potential for brain shift after the subarachnoid space is entered and CSF egress occurs. This consideration is especially germane in AD patients, who usually have substantial brain atrophy and consequent enlargement of the sub-

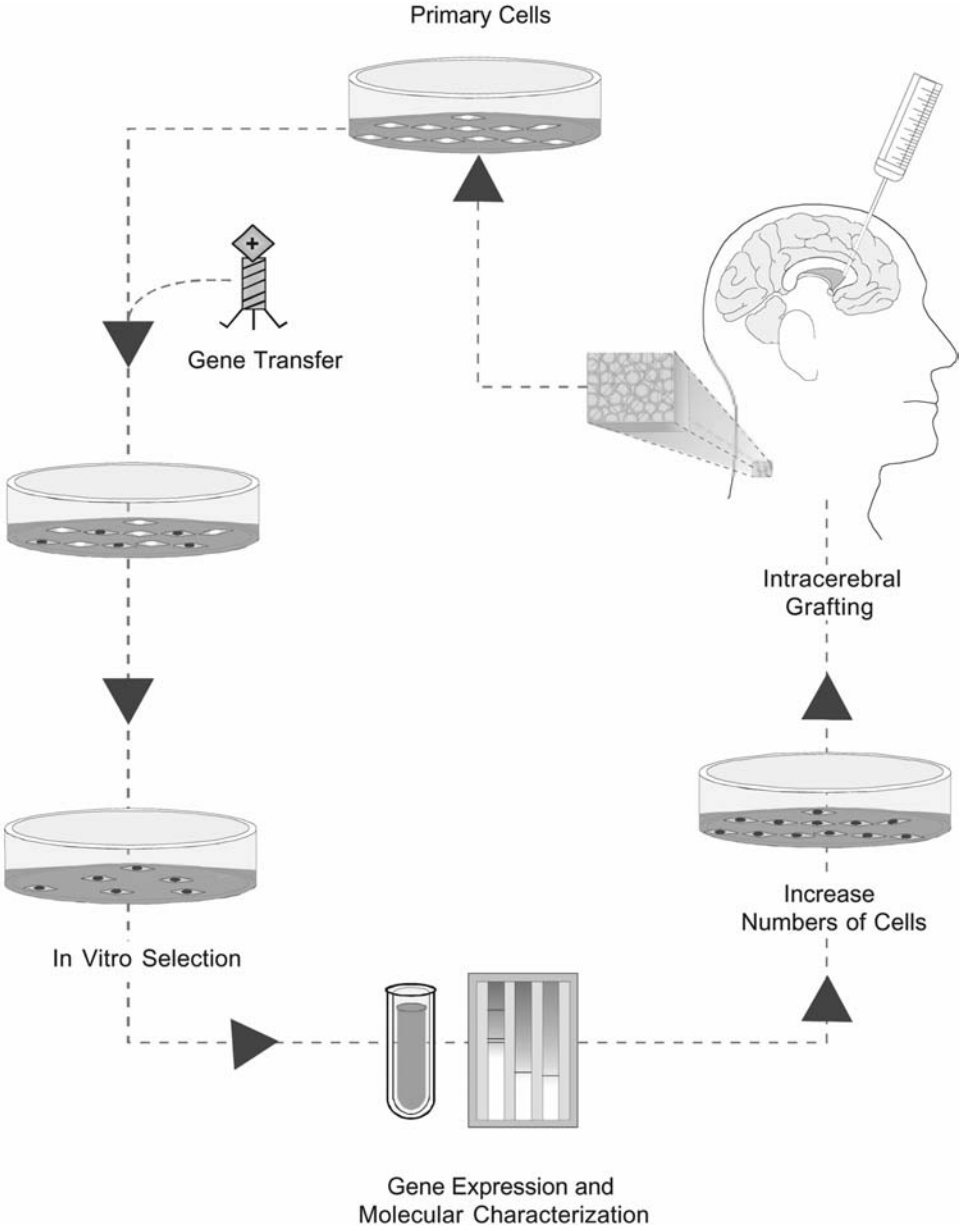

Figure 2 Schematic illustrating the basic steps in ex vivo gene therapy. See the color insert for a color version of this figure.

arachnoid space. To minimize this source of error, during surgical implantation every attempt must be made to minimize CSF egress through the bur hole. Design of the surgical procedure must also take into account the shape and orientation of the basal nucleus of Meynert. The NBM exhibits an anterior/mesial/ventral to posterior/lateral/dorsal orientation between the anterior commissure and the base of the brain. This orientation does not permit a single needle passage, depositing implanted cells along the axis of the needle, to populate the entire NBM with grafted cells. Multiple deposits therefore have to be made by passing the injection needle from the cortex to the target along multiple separate tracts. This increases the risks of hemorrhage as well as potential risk of damage to nerve fiber tracts. To decrease this risk, the planning of the operative procedure must involve planning a trajectory that does not require the needle to traverse the ventricles or any major groups of vessels. In addition, multiple injections are mandated by the fact that NGF secreted by the engineered fibroblasts exhibits limited diffusion into the surrounding brain. The entire operative procedure must also be undertaken

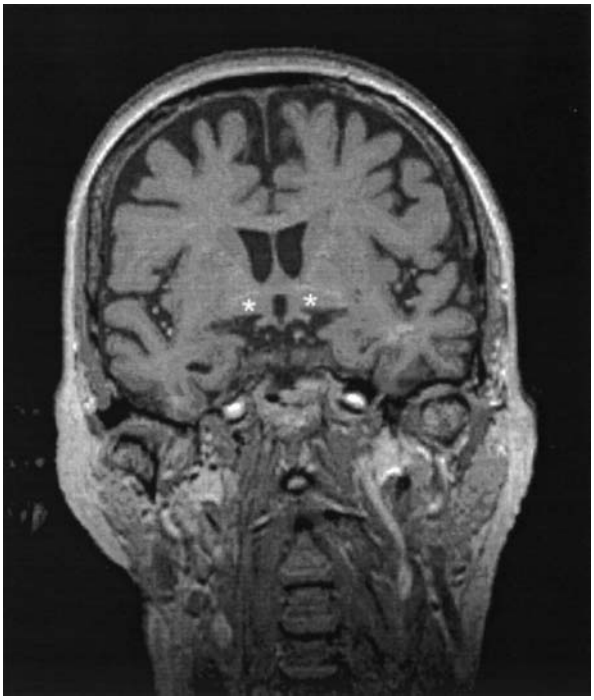

Figure 3 A coronal cut T1-weighted MRI at the level of the basal forebrain nuclei. Note the diffuse cerebral atrophy characteristic of AD. Asterisks indicate the anteriormost part of the basal forebrain nuclei. The anterior commissure is seen crossing just above the asterisks.

under deep enough anesthesia to prevent movement of the patient because trauma to the brain could occur if the head and brain move while the injection needle is inside the brain. Last, the injected cells are genetically altered and the potential for tumorigenic transformation exists. The ability to visualize the implant after injection would be essential to detect any growth of the graft. Provision for elimination of any tumor that may form (e.g., Gamma Knife radiosurgery) must be in place prior to clinical application.

These considerations must be accounted for in the design of any clinical trial of ex vivo gene therapy for AD. Such a clinical trial is currently in progress (48).

IV. FUTURE DIRECTIONS

We anticipate that in the future improved delivery techniques will make the process of factor/gene introduction safer. At present, the injection catheters are passed blindly, with a small but real risk of hemorrhage. In the near term, improved delivery will depend on further refinements in surgical instrumentation and technique and better imaging. Safety could be improved with real-time MRI and the ability to see potential hazards in the brain, such as blood vessels. The development of a readily available, renewable cell stock, preferably that does not require harvest from the individual to be transplanted, is also desirable. In vivo regulatable gene expression constructs would represent a significant advance in our ability to control the implants. Blesch et al. demonstrated the feasibility of this strategy using in vitro modulation of neurite outgrowth using rat fibroblasts transduced with a tetracycline-repressible construct secreting NGF (49). For a chronic disease such as AD, constitutive expression of NGF is probably appropriate. However, given that grafts may survive and produce NGF with variable success in different individuals, regulatable implants would allow a clinician to titrate the amount of trophic factor output to the needs of the patient.

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Prospects for Clinical Application of Synthetic Gene Delivery Vectors

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I. INTRODUCTION

In many respects synthetic (''nonviral'') gene transfer vectors, comprised of either naked DNA or DNA complexed with a cationic lipid or polymer, are in a formative stage of development for use in vivo. Several diverse chemical compounds and physical methods of gene delivery are under evaluation, the mechanisms of gene transfer into different tissues are still being elucidated, and the acute and long-term toxicological consequences in vivo have not been fully characterized. Thus, it is perhaps surprising that almost one-fourth of the clinical gene therapy trials to date have used synthetic vectors, involving more than 700 patients (Fig. 1A). However, the overwhelming number of these protocols has been for the treatment of different cancers and far fewer noncancer trials have been initiated (Fig. 1B, Table 1). This chapter reviews some of the recent preclinical and clinical applications of synthetic vectors, highlighting a few disease targets that are representative of current synthetic gene delivery strategies. Included will be some discussion on the realistic prospects for synthetic vectors to be an effective therapeutic for a given disease indication and the technical challenges remaining to achieve that goal.

A. Current Status of Synthetic Vector Systems

What is the present state of synthetic gene transfer technology? Many of the originally perceived advantages of synthetic vectors are still valid, perhaps foremost being that synthetic vectors are not viruses, and as such they avoid most (but not all) of the immune complications associated with recombinant viral vectors. Although viruses have evolved over the millennia to evade host defenses, the host has likewise evolved a sophisticated system to recognize and eliminate or neutralize foreign pathogens. Because synthetic vectors presumably do not provoke specific immune responses, in theory they could be administered repeatedly, and are therefore conducive for treating diseases requiring prolonged or even lifetime correction. However, a number of overriding limitations of synthetic vectors have so far precluded capitalizing on this important feature.

One of the most significant problems continues to be the efficiency of gene transfer, and therefore the level of transgene expression that can be realized. The best synthetic vector systems express at levels that are generally 10 to 1000-fold lower than can be generated from recombinant viral vectors. This severely reduces the number of diseases for which synthetic vectors can be expected to generate therapeutic levels of a given protein. Numerous cationic liposomes, molecular conjugates, polymeric carriers, and peptides have been synthesized that mediate efficient transduction in tissue culture cells (1,2). Electroporation and ultrasound have been reported to enhance the efficiency of naked DNA transduction in skeletal muscle and other tissues (3). Novel physical methods of gene delivery have also been developed, such as catheter-mediated delivery of plasmid DNA (pDNA) to the liver and intravascular delivery into limb muscles under pressure (Fig. 2) (4–6). These techniques can generate levels of transgene expression that are significantly higher than some viral vectors such as adeno-associated virus (AAV). However, use of these methods in a clinical setting will require a further evaluation of their practicality and safety, including studies in nonhuman primates (7). At present, synthetic gene transfer efficiency remains a significant barrier to many clinical applications, but this limitation may be overcome with improved formulations and delivery methods.

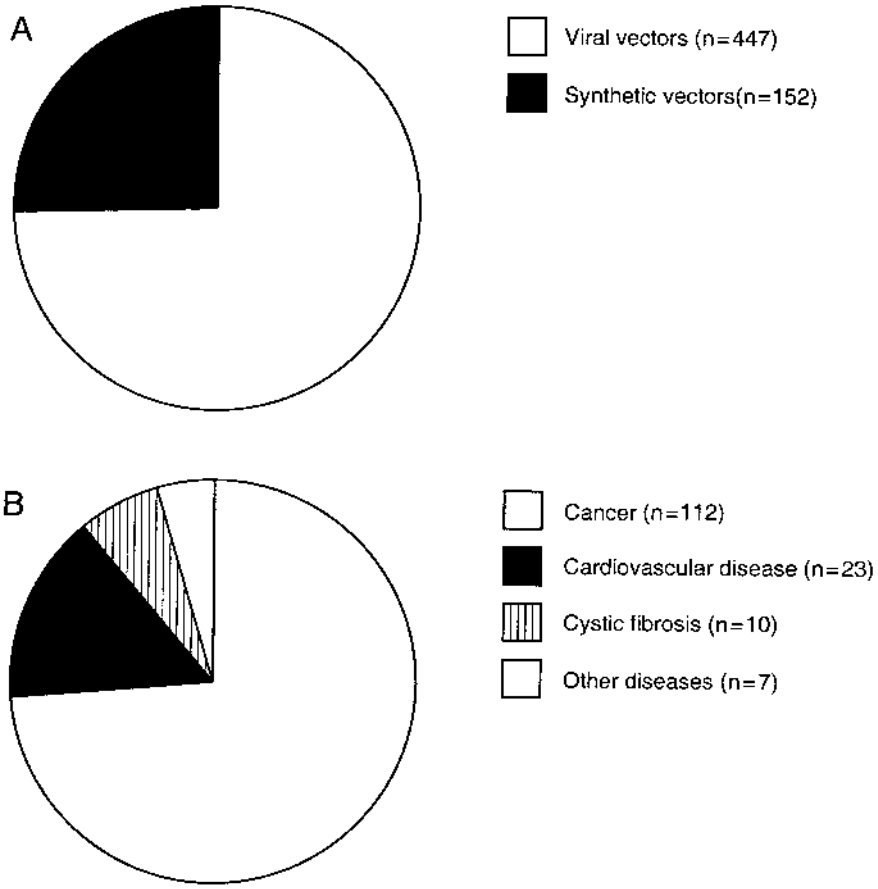

Figure 1 (A) Gene transfer vectors used in clinical trial protocols. (B) Clinical trial protocols using synthetic vectors. (Adapted from Ref. 93.)

Table 1 Clinical Trials Using Synthetic Vectors

Disease	Gene	Vector	Delivery	No. trials
Cancer	HLA-B7/β 2-microglobulin, IL-2, E1A, GM-CSF, gp100, others	Naked DNA, complex	Intratumoral, intraperitoneal, gene gun	112
Cardiovascular disease	VEGF, FGF, Del-1, others	Naked DNA, complex	Intramuscular	23
Cystic fibrosis	CFTR, AAT	Complex	Intranasal, aerosol	10
Bone disorders/fractures	Parathyroid hormone	Naked DNA	Bone implant	2
Cubital tunnel syndrome	IGF-1	Naked DNA	Intramuscular	1
Rheumatoid arthritis	HSV-TK	Naked DNA	Intraarterial	1
Hemophilia A	Factor VIII	Naked DNA	In vitro to skin fibroblasts	1
Canavan disease	Aspartoacylase	Complex	Intracranial	1
Alpha-1-antitrypsin deficiency	AAT	Complex	Intranasal	1

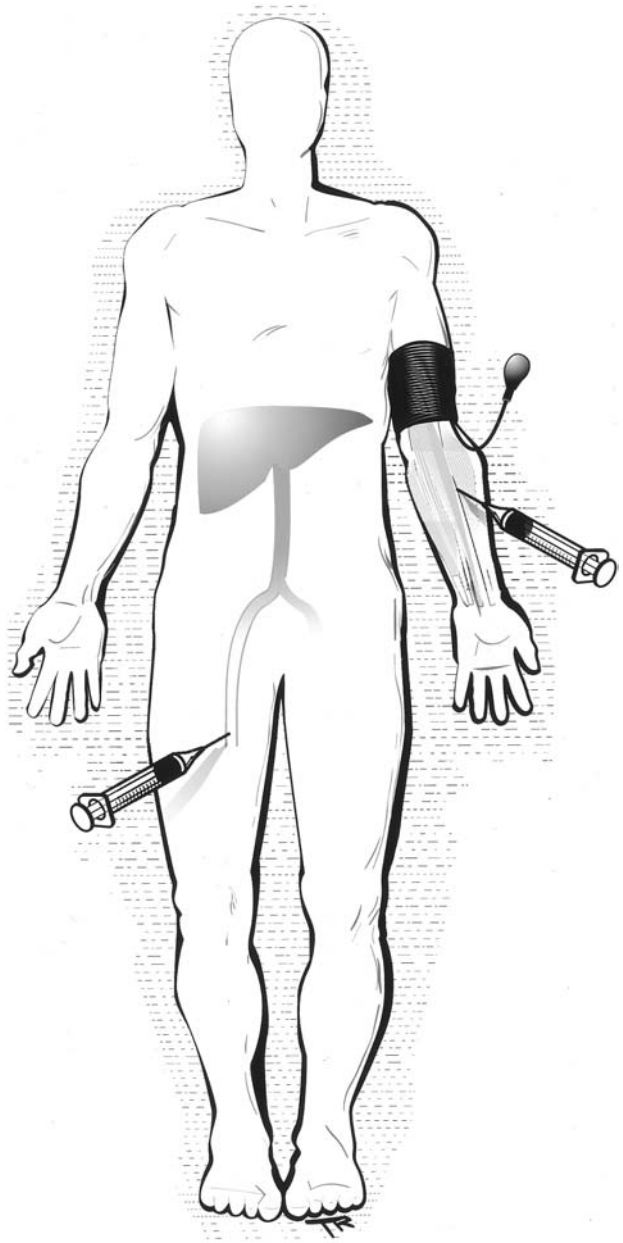

Figure 2 Two possible clinical approaches for delivering naked DNA. Depicted are intravascular delivery of DNA into skeletal muscle and catheter-mediated delivery of DNA into the liver.

Transient expression has long been another defining characteristic of synthetic vectors, which has until recently limited potential clinical applications to acute therapies. However, there are now examples of long-term expression from synthetic vectors lasting several weeks to greater than 1 year in vivo. A key aspect has been to identify promoters that remain transcriptionally active over an extended period in the major target organs, such as muscle, lung, and liver. Part of that challenge has been to identify promoters or promoter hybrids that are as transcriptionally robust as that of the widely used human cytomegalovirus (CMV) promoter, while not being prone to the known observed inactivation of CMV. Combinations of liver-specific promoter and enhancer elements have been identified that confer sustained expression in the liver with levels equivalent or superior to that of CMV (8,9). Hybrid promoters containing viral enhancer sequences linked to cellular promoters such as β-actin or ubiquitin also have been demonstrated to have significantly increased duration of expression (10,11).

Other advances in increasing the persistence of expression include the use of integrating elements to facilitate the retention of pDNA in tissues, for example, transposons (12) and phage integrases that promote integration of the vector (13,14). In addition, pDNA vectors that have been largely depleted of CpG motifs confer sustained expression through a mechanism that is presently unknown (15). Taken together, synthetic vectors are no longer limited to acute therapies and can be considered for diseases requiring long-term correction.

One of the more serious problems of synthetic vectors that are composed of a complex of DNA and a cationic liposome, polyamine, or other polycation is the acute inflammatory response observed in vivo. Intratracheal or intranasal delivery of cationic lipid–pDNA complexes induces an acute, dose-dependent inflammatory response characterized by an influx of neutrophils and the elevation of proinflammatory cytokines such as IFN-γ, TNF-α, IL-6, and IL-12 (16). Intravenous injection of complex causes a similar induction of cytokines as well as a dose-dependent activation of complement, loss of lymphocytes and platelets, and elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels indicative of liver damage (17). Injecting higher doses of complex can lead to significant morbidity. Several groups have shown that immunostimulatory CpG motifs within the pDNA vector play a significant role in the acute inflammatory and toxic response to cationic lipid–pDNA complexes (18–20). Methylation of the CpGs in the pDNA can markedly reduce cytokine induction, but interestingly cannot eliminate other toxic parameters such as lymphocyte and platelet loss or elevation of liver enzymes (21). Only synthetic pDNA vectors that have been largely depleted of CpG motifs can reduce but not totally obviate the acute toxic response (15).

B. Disease Targets for Synthetic Vectors

Given the limitations of the current technology, certain diseases are clearly more suitable for synthetic vectors than others. Cancer is highly amenable to treatment with synthetic vectors because many of the approaches involve either direct intratumoral injection or ex vivo modification of tumor cells or antigen-presenting cells, which avoids the problems associated with in vivo transduction. Diseases requiring only low levels of a secreted protein would also be favored targets given the low efficiency of gene transfer and achievable levels of expression. Such diseases also permit the muscle or lung to serve as the source of protein that can be secreted into the

circulation and distributed to other organs. Diseases that can be treated by cytokines, hormones, or growth factors therefore may be less challenging than diseases requiring correction of a protein deficiency.

II. SYNTHETIC VECTORS FOR INHERITED GENETIC DISORDERS

One class of disorders being targeted with synthetic vectors are those that are caused by a single gene mutation. Of the numerous diseases being targeted, reviewed here will be only a small representative subset that uses various vector systems and routes of delivery. In 3 of the diseases (cystic fibrosis, α-1-antitrypin deficiency, and Canavan disease), 1 or more clinical trials have already been conducted.

A. Cystic Fibrosis

Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic adenosine monophosphate (AMP) activated chloride channel (22). This protein resides in the apical membrane of secretory epithelia, and in the lung is essential for maintaining proper fluid and electrolyte balance. Defects in CFTR function lead to accumulation of thickened mucus in the airway, bacterial colonization, and chronic pulmonary inflammation (23). Consequently, most CF patients die from lung failure.

Synthetic vectors have been considered appropriate for CF in part because the lung is the primary target organ, and thus the vectors can be delivered locally into the airway in the form of an aerosol without the need for targeting. Another consideration is that CF patients will have to be treated for their entire lifetimes. As such, repeat administration will be necessary, which in the absence of immunosuppression regimes is problematic for viral vectors. The interval between doses may be as often as every few months, given the normal turnover of airway cells and the increased rate of turnover in the inflamed CF lung. Preclinical studies with synthetic vectors have demonstrated correction of the CFTR-mediated chloride transport defect in vitro, and CFTR expression in the airways after aerosol delivery of cationic lipid–pDNA complexes. The levels of expression were quite low and transient, but nevertheless several groups moved forward to phase I trials in humans, primarily to assess safety.

There have been 10 clinical trials using synthetic vectors for CF (24–29). In the initial trials, cationic lipid–pDNA complex was delivered to the nasal epithelium, which served as a safe, easily accessible surrogate for the cells lining the pulmonary airway. The complex was applied using a syringe or spray, in some instances, over a period as long as 7 h (29). There was no evidence of inflammation or toxicity as a result of these treatments. Plasmid DNA and vector-specific RNA were detected in a proportion of the samples analyzed, and an increase in CFTR-mediated chloride conductance was observed in some trials, but not in others. No significant change in basal potential difference (PD), a measure of the sodium transport defect that is also present in CF patients, was observed in any of the trials. The results satisfied the primary goal of the trials showing that delivery of complex to the nose was safe.

Trials were also conducted in the lung using aerosolized cationic lipid–pDNA complex. The lung trials used a single cationic lipid (GL-67) and a CMV promoter-based plasmid that expressed wild-type CFTR. Although no changes in basal PD measurements were observed, Alton et al. (24) measured an in vivo potential difference toward normal values in 6 of 8 patients that received complex. This correction was considered statistically significant and represented a 25% restoration of chloride channel function. There was also evidence of decreased bacterial adherence on the treated airway epithelial cells.

These encouraging results were somewhat tempered by the observation of an acute inflammatory response to the administered complex, which was characterized as mild in 1 trial (24), but more severe in a second trial (26). In both, the patients developed flulike symptoms that included myalgia, headache, and fever, that began within 6 h and resolved within 24 to 48 h after treatment. This response was not observed when the same cationic lipid formulation without pDNA was aerosolized into the lungs of normal volunteers, suggesting that the complex of cationic lipid with pDNA was the causative agent (30). Unmethylated CpG motifs within the pDNA have been shown to contribute most (but likely not all) of the acute inflammation observed in mice that received complex intranasally. However, it remains to be determined whether immunostimulatory CpG motifs were the cause of the transient flulike symptoms in the human studies.

There are several challenges to be overcome before synthetic vectors can provide bonafide clinical benefit to CF patients. Many of these problems pertain to both viral and nonviral gene therapy approaches for CF (31). One barrier to gene transfer is the highly viscous mucus overlying the CF lung epithelium. Second, chronic airway infections induce inflammation, which is known to down-regulate gene expression of some promoters. Third, CFTR is not a secreted protein and will require transduction of at least 10% of the airway epithelial cells to restore normal chloride transport (32). Finally, clear and distinct measurements of clinical efficacy remain problematic (29,33).

Various strategies have been formulated to address these issues. Mucolytic agents have been shown to reduce the mucous barrier and increase gene expression (34). Promoters such as ubiquitin appear to have decreased sensitivity to inflammation and confer sustained expression in the lung (10,11). Repeated administration of a synthetic vector that has persistent expression may incrementally increase the percentage of cells transduced (Fig. 3). However, the validity of this approach must still be demonstrated and gene transfer efficiency will likely remain a significant challenge for CF therapy using synthetic vectors.

B. Alpha-1-antitrypsin Deficiency

Another lung disease that can potentially be treated with synthetic vectors is α-1-antitrypsin (AAT) deficiency. AAT defi-

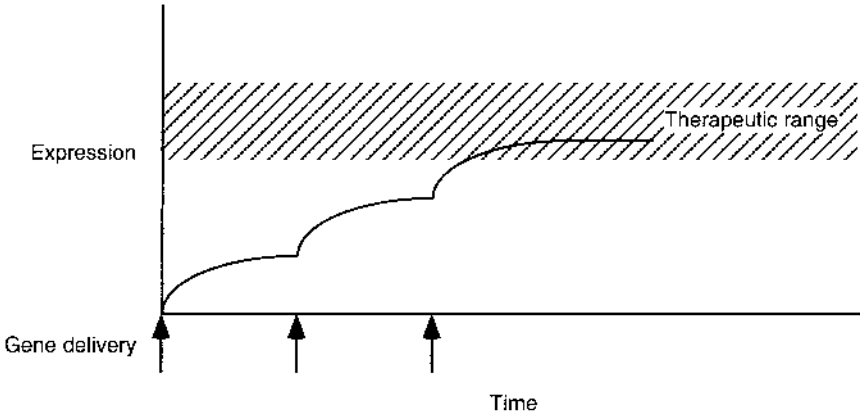

Figure 3 Theoretical strategy for achieving therapeutic levels of expression from synthetic vectors. Multiple dosing using a pDNA vector that confers sustained expression may lead to cumulative expression over time.

ciency is an autosomal recessive disorder caused by mutations in the gene encoding AAT, the principal antiprotease present in human plasma. AAT inhibits neutrophil elastase and thereby helps to protect the lung from destruction by this protease. Patients with low levels of AAT are susceptible to developing emphysema, and a subset also develops liver disease (35).

One gene therapy strategy for AAT deficiency is to transduce the liver, the normal site of AAT synthesis. The AAT is then secreted into the circulation and taken up by the lung (36). A second approach is to deliver the gene directly into the lumen of the lung, taking advantage of the ability of cationic lipid–DNA complexes to transduce the lung parenchyma. With this strategy lower but localized increases in AAT concentration may be as or more effective than elevating serum AAT levels. The levels of AAT required to be protective are not known, but in vitro studies have shown that small changes in AAT concentration can lead to relatively large alterations in neutrophil proteolytic activity (37).

A clinical trial was conducted on 5 patients with AAT deficiency (38). The cationic lipid formulation DOTMA-DOPE was complexed with a pDNA vector expressing AAT. As in the CF trials, the nose was used as a surrogate for the lung, and complex was applied to one nostril with the other nostril serving as the control. AAT levels in the nasal lavage fluid increased about 2-fold over baseline, peaking at day 5 and returning to near baseline levels by day 14. Although the peak levels of AAT reached only one-third of the normal mean, there appeared to be an anti-inflammatory effect from the treatment as evidenced by a slight but statistically significant decrease in IL-8 levels in the nasal lavage fluid. In contrast, intravenous delivery of AAT protein resulted in levels of AAT in the nasal lavage fluid within the normal range, but IL-8 levels did not decrease. These results bolster the contention that certain critical regions (e.g., the interstitial spaces between cells) are more easily accessible using localized gene transfer rather than intravenous delivery of protein (38).

If these studies are to progress to the lung, the same safety issues as in CF will need to be addressed, namely the acute inflammatory response from aerosolized cationic lipid–pDNA complex. Also as in CF, the endpoints for demonstrating clinical efficacy may be problematic. Measuring significant decreases in the rate of decline of lung function or the frequency of lung infections will require monitoring a large number of patients over a significant period of time. On the favorable side, complex can be aerosolized to reach the desired alveolar region and the levels of AAT needed to be protective may be low. This would argue for the use of a lower dose of complex that would then minimize any inflammatory response associated with the vector.

C. Lysosomal Storage Disorders

Another potential target for synthetic vectors are lysosomal storage diseases (LSDs), which comprise a group of more than 40 different disorders caused by deficiencies in different catabolic enzymes present in the lysosome that normally breakdown glycoproteins, glycolipids, and other macromolecules (39). As a result there is abnormal accumulation of intracellular storage products that, depending on the nature of the macromolecule, leads to dysfunction of 1 or more organs. LSDs are favorable targets for synthetic vectors because the threshold to correct the disease phenotype is predicted to be low, approximately 5% to 10% of normal levels based on milder forms of some LSDs. Second, a proportion of the lysosomal enzymes are secreted from the cell and are capable of being taken up by distant cells. This suggests that an organ such as the lung, liver, or skeletal muscle can be transduced to secrete a given lysosomal enzyme into the circulation that can then be distributed throughout the body.

Efforts using synthetic vectors to treat LSDs have been very preliminary to date. Marshall et al. (40) constructed pDNA vectors that express glucocerebrosidase, the enzyme that is deficient in Gaucher disease (41). The deficiency leads to an accumulation of the glycolipid glucosylceramide (GL-1) in tissue macrophages. The pDNA vector was injected hydrodynamically into the livers of a chemically induced mouse model of the disease, in which the mice transiently accumulate high levels of GL-1 within the lysosomes of their Kupffer cells. The injected mice expressed elevated levels of glucocerebrosidase in their livers and serum, and the levels of GL-1 levels were observed to decrease to baseline.

Synthetic vectors have also been used to treat a mouse model of Fabry disease, an X-linked recessive disorder caused by a deficiency of α-galactosidase A, which leads to an accumulation of the glycosphingolipid globotriaolsylceramide (GL-3) in the vascular endothelium of most tissues of the body. Fabry knockout mice have been injected intravenously with a cationic lipid complexed to a CpG-depleted pDNA vector (42). As expected, the highest level of α-galactosidase A expression was in the lung, with much lower but detectable levels in serum and the other organs. Expression persisted for approximately 2 months. During this time GL-3 levels declined gradually by 20% to 50% in the lung, liver, and heart. However, there was no reduction of GL-3 levels in the kidney, which is a critical target organ in Fabry patients.

Although GL-3 levels were only partially reduced in these studies, the results suggest that even very low but sustained expression may be sufficient to gradually deplete from the lysosome an accumulated glycolipid or glycoprotein. The transduced enzyme activity would only need to be slightly greater than the rate of accumulation of a given macromolecule. However, some of the challenges that remain include (1) the toxicity of systemically delivered complexes, (2) delivering enzyme to tissues that are difficult to reach from the circulation (e.g., bone to correct the skeletal defects in mucopolysachharidosis type VII), and (3) the need to correct both the central nervous system and visceral organs for many LSDs. Perhaps most important, the levels of expression in the lung and the amount of enzyme secreted into the circulation are at present marginally therapeutic. Improving secretion of the enzyme from the lung or targeting complexes to the liver may be beneficial. Repeat dosing may also cumulatively increase expression levels.

D. Familial Hypercholesterolemia

Synthetic vectors are being evaluated for the treatment of familial hypercholesterolemia (FH) and hyperlipidemias. FH is an autosomal dominant disorder caused by mutations in the low-density lipoprotein (LDL) receptor. Patients have persistently elevated levels of LDL in their serum, which leads to the development of atherosclerosis and coronary artery disease (43). Tomita et al. (44) injected a complex of hemagglutinating virus of Japan (HVJ) liposomes and a plasmid expressing the human LDL receptor into the hepatic portal vein of LDL receptor knockout mice. RNA was detected in the livers that peaked at days 7 to 10 after injection, but became undetectable by day 21. At day 7 there was a modest but statistically significant decrease in total cholesterol levels. Another approach being taken to treat hypercholesterolemia is to express apolipoprotein E, which is involved in removing excess lipoproteins and cholesterol from tissues. Rinaldi et al. (45) injected a plasmid that expresses apolipoprotein E (apoE) into the muscles of apoE-deficient mice. Very low levels of apoE (0.6 ng/mL) were produced in the serum, but this was sufficient to decrease serum cholesterol levels by 25% to 43%, and this reduction persisted for 16 weeks. Athanasopolulos et al. (46) also injected an apoE plasmid into apoE-deficient mice, but did not detect measurable levels of apoE in serum nor a decrease in serum cholesterol levels. Nevertheless, they did observe a retardation in the development of atherosclerotic and xanthomatous lesions.

The above results suggest that the threshold for successfully treating at least some aspects of hypercholesterolemia may be quite low, requiring expression of less than 1% of normal apoE levels to realize some benefit. This concept is supported by studies in transgenic mice expressing apoE from adrenal glands where 1% to 2% of normal serum apoE levels did not reduce hyperlipidemia but inhibited atherosclerosis (47). However, it has also been demonstrated in studies using adenoviral or AAV vectors that higher levels of expression will confer greater protection against atherosclerosis. For example, a helper-dependent adenoviral vector expressing a 1000-fold higher level of apoE in the serum than plasmid vectors inhibited the formation of atherosclerotic lesions for greater than 2 years after a single injection (48). These results suggest that higher and more sustained expression from synthetic vectors is desirable for treating FH and other hyperlipidemias.

E. Neurological Diseases—Canavan Disease

Canavan disease is an autosomal recessive disorder caused by a deficiency in the enzyme aspartoacylase, resulting in an accumulation of N-acetyl-aspartate (NAA) in the brain. The accumulation of NAA in the white matter causes spongiform degeneration of the brain, leading to severe neurological and motor impairment (49,50). Symptoms arise during infancy, and the disease is uniformly fatal.

Leone et al. (51) conducted preclinical studies in rats using LPD particles, which consist of the cationic lipid formulation DC-Chol/DOPE, the polycations poly-L-lysine or protamine sulfate, and pDNA. Intraventricular injection of LPD particles using a LacZ reporter plasmid showed (by LacZ staining) sustained expression for 10 months. Injection of LPD complex using a NAA plasmid in 2 monkeys showed no apparent toxicity, and NAA transcripts were detected by RT-PCR at 1 month.

Based on these preclinical studies, a phase I clinical study was then conducted in 2 children with Canavan disease (51). Five milliliters of LPD complex containing 400 μg of pDNA was injected into the lateral ventricle using a catheter. NAA levels were measured noninvasively and shown to be within

the normal range in the frontal and parietal, but not the occipital lobe 1 month after treatment. The levels in 2 of the lobes remained within the normal range in 1 patient for 12 months. There were some radiological, neurological, and behavioral improvements; however, it was not possible to draw firm conclusions regarding efficacy because of the small sample size and unblinded nature of the study.

Despite these early clinical efforts, application of synthetic vectors to neurological diseases will require greatly improved formulations that are optimized for gene transfer into the brain, and more extensive studies in animal models. Because of the equivocal and transient clinical results using synthetic vectors, Leone and her colleagues (52) switched to AAV vectors for their clinical trial that began in 2001.

III. SYNTHETIC VECTORS FOR ACQUIRED DISEASES

A second class of disorders being targeted with synthetic vectors are those that are not caused by a single gene mutation, but rather have a more complex etiology. Examples considered here are melanoma, myocardial ischemia, diabetes, and chronic wounds.

A. Melanoma

As seen in Table 1, by far the largest number of clinical protocols using synthetic vectors are for different cancers. This is largely a reflection of the preponderance of cancer clinical trials irrespective of the vector system used. Several different therapeutic strategies are being evaluated with synthetic vectors, including the use of antioncogenes, prodrug activating genes, tumor suppressor genes, and immunotherapeutic genes (53–56).

Several arguments can be made for using synthetic vectors for cancer. First, direct intratumoral injection is simple, minimizes systemic toxicity, and can be repeated multiple times, if needed. Second, cationic lipid–pDNA complexes that are delivered systemically or intraperitoneally are able to transduce tumors. Third, immunotherapy strategies require only relatively transient expression, sufficient to activate an immune response to the tumor. Last, immunostimulatory CpG motifs within the pDNA vector have been shown to stimulate a potent cytotoxic T lymphocyte (CTL) response, which is deleterious for most disease targets, but can be beneficial in activating an immune response to the tumor (57). In fact, several studies have shown that pDNA in the absence of expression of any cDNA is sufficient to induce tumor regression and increase survival in mouse models (58–61).

Although the preclinical and clinical studies using synthetic vectors for melanoma are too numerous and varied to be reviewed here, a few illustrative clinical trials using immunotherapy to treat melanoma can be described briefly. Advanced stages of melanoma are difficult to treat because of the development of metastatic lesions. In contrast, melanoma is characteristically highly immunogenic, and so is more responsive to immunotherapeutic approaches than many other cancers. Several groups have sought to express an allogeneic HLA-B7 molecule in a given tumor to promote a CTL response to the tumor (62). Cationic lipid–pDNA complexes were injected directly into the tumors of patients with advanced (stage IV) melanoma, with some patients receiving multiple injections at weekly intervals. Biopsies of the treated tumor were shown to contain vector-specific DNA and RNA, and enhanced tumor infiltrating lymphocyte activity was seen in most patients. In one trial, 7 of 17 patients had tumor regression of 25% or more (63). In another trial, 1 of 5 patients showed complete remission, with regression of the 2 injected nodules and distant metastatic lesions that were not injected (64).

The results of the clinical studies indicate that intratumoral injection of complex is safe and a clear clinical response was observed in some patients. However, it is at present unclear why tumor regression was seen in only a subset of the patients treated. A number of other approaches are under investigation, including the use of irradiated autologous tumor cells or dendritic cells transfected with cytokines or tumor-specific cell surface antigens (65–67). These efforts are directed toward generating a more robust immune response to the tumor and enhance response rates. Further advances in understanding tumor immunity combined with more efficient synthetic gene delivery vectors should improve the prospects for an effective immunotherapy for melanoma.

B. Myocardial Ischemia

Second to cancer indications are clinical protocols for cardiovascular disease, and most of these have used synthetic vectors to promote therapeutic angiogenesis for the treatment of cardiac ischemia. This condition, characterized by insufficient blood flow to the heart, oxygen deprivation, and inadequate removal of metabolites, is usually caused by the presence of atherosclerotic plaques in the blood vessel. Different growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-1 and FGF-2), and hepatocyte growth factor can stimulate the development of collateral vessels and improve myocardial perfusion (68).

The attractive feature of ischemia as a disease target for synthetic vectors is that these growth factors are highly potent. Expression levels can thus be relatively low and transient as well, just sufficient to promote vessel growth in the affected tissue. VEGF is also a naturally secreted protein and can be distributed to neighboring cells. In addition, the target tissue, cardiac or skeletal muscle (in the case of limb ischemia) is amenable to transduction by naked DNA.

A number of clinical trials have been performed on patients with end-stage coronary heart disease, involving injection of VEGF pDNA vectors at multiple sites into the myocardium. No adverse changes in heart function were observed as a result of the injections. Signs of clinical efficacy, such as decreased angina, were observed in many of the early trials, although interpretation was difficult in the absence of a placebo control group. A more recent randomized, double-blind, placebo-con-

trolled trial of 19 patients had results consistent with earlier trials. The patients exhibited improved exercise tolerance, had fewer episodes of angina, and required less medication (69).

The results show promise for further clinical development of synthetic vectors for ischemia. Gene transfer efficiency is low, but may be augmented using various physical methods (e.g., ultrasound, electroporation). In addition, other transgenes may be used in place of or in conjunction with VEGF to promote angiogenesis. One such transgene encodes a constitutively active hybrid transcription factor, HIF-1α/VP16 (70). HIF-1α regulates the expression of a large number of genes involved in O_2 homeostasis, including VEGF. In an acute myocardial infarction model in the rat, the combination of VEGF and HIF-1α/VP16 generated a higher capillary density than either factor alone (71).

C. Diabetes

Type I diabetes is an autoimmune disorder in which the insulin-secreting β cells of the pancreas are eventually completely destroyed. The lack of insulin results in elevated levels of glucose in the blood. Unless glucose levels are strictly controlled, patients suffer from complications such as blindness, heart and kidney disease, neuropathy, and limb ulcers.

Synthetic vectors expressing insulin have been evaluated in the streptozotocin-induced diabetic mouse model, where the goal has not been to completely restore insulin levels, but rather to provide basal levels of insulin as an adjunct to current insulin treatments. Morishita et al. (72) injected HVJ liposomes and an insulin expression plasmid to SZT-induced diabetic mice. Serum insulin levels were quite low (2 μU/mL at day 4) and persisted for only 2 weeks, although repeat injections increased the duration of expression. A number of groups have used skeletal muscle to secrete insulin by injecting naked pDNA vectors containing a furin-cleavable proinsulin cDNA, which in muscle is processed to mature insulin by endogenous furin (73–75). The peak of serum expression occurred approximately 1 week after injection and expression persisted for 4 to 6 weeks. Martinenghi et al. (76) also performed in vivo electroporation of the muscle and achieved some of the highest levels of insulin in the serum, peaking at 68 μU/mL. Furthermore, while the untreated streptozotocin-treated mice died within 3 weeks, the treated mice survived up to 10 weeks.

Clinical application of this muscle depot approach will require translation of these results into larger animal models, which may be problematic as the efficiency of transducing skeletal muscle has been observed to decrease as the size of the animal increases. Vascular delivery of pDNA under pressure may be an alternate strategy (6). A second requirement will be the need for some sort of regulated expression to avoid the toxicity of unregulated insulin levels.

D. Chronic Wounds

The treatment of chronic wounds is an attractive candidate for synthetic vectors. Problematic nonhealing wounds can be caused by burns, diabetes, pressure, vascular disease, or connective tissue disease, and represent a major clinical problem that leads to significant morbidity.

There are several arguments for using gene therapy to treat chronic wounds (77,78). The skin is readily accessible for gene delivery, and at the same time vector delivery and expression can be localized, reducing systemic toxicity (79). The effectiveness of treating lesions with growth factors has been limited by the high cost of the purified factors, the short half-lives of the proteins due to the presence of high concentrations of proteases in the wound, and the potential toxicity from the large doses required. Gene therapy could provide more constant levels of growth factor locally, the expression of which could be of relatively short duration that is sufficient for closure of the wound.

Several growth factors have been shown to promote wound healing. Those factors that have been expressed from pDNA vectors include platelet-derived growth factor, keratinocyte growth factor, insulin-like growth factor-1 (80,81), acidic fibroblast growth factor (82), epidermal growth factor, and transforming growth factor β-1. The choice of which factor to use may depend on the type of wound and the stage at which the wound is treated.

These plasmids have been delivered to the wound site by 3 principal methods. One approach is particle-mediated gene transfer involving high-velocity delivery of pDNA adhered to gold particles (83). This technique has been used to express EGF, PDGF, and TGFβ-1 in wound sites and shown to accelerate healing and increase wound tensile strength. A second approach is subcutaneous injection of liposomal–pDNA complexes. Jescke et al. (84) demonstrated that injection of a complex of DMRIE-Chol and a plasmid expressing KGF into thermally injured rats greatly improved epidermal and dermal regeneration. A third approach has been to embed pDNA within a gel or matrix (Fig. 4) (85,86). Cells are able to migrate into these matrices and take up the pDNA. For example, a pDNA expressing platelet-derived growth factor embedded in a collagen matrix has been shown to promote wound healing in a dermal ulcer model (87).

Although viral vectors such as adenovirus (88) and AAV (89) have also been evaluated, this is one application where nonviral delivery methods are prominent. Efficiency of gene delivery remains an issue, as it has been shown that administration of too high a dose of pDNA will actually inhibit the rate of wound healing (90,91). More efficient physical delivery methods, such as microseeding (92), improved liposomal formulations, and more effective polymer matrices will improve efficacy. Combinations of growth factors may be beneficial, and regulated expression systems need to be considered. Safety issues for this application seem to be minimal, and overall, clinical use of synthetic vectors for wound healing appear to be quite promising.

IV. SUMMARY

Synthetic vectors have been applied to a wide range of inherited and acquired diseases, and this review has covered only

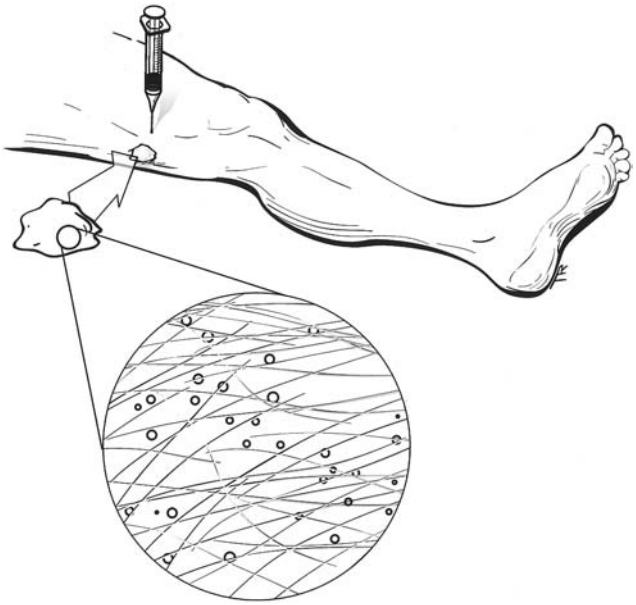

Figure 4 Synthetic gene delivery to promote wound healing. Cationic lipid–pDNA complexes can be injected subcutaneously at sites near the wound margin. Alternatively, pDNA can be embedded in a collagen or fibrin matrix. Cells migrating into the matrix take up the pDNA and express a given soluble factor.

a small list of disorders for which synthetic vectors have been used. Efficacy has been demonstrated in several animal models and clinical trials have been conducted. The preclinical and clinical results revealed genuine promise but also definite limitations that should perhaps serve as a cautionary sign for moving synthetic vectors too aggressively into clinical use.

Safety is of prime concern for any gene therapy, and although synthetic vectors are generally safe, an acute inflammatory response is observed with certain vector systems and delivery routes. Naked DNA delivery into muscle appears to present the least number of complications, with a minimal (but not totally benign) inflammatory response and little to no systemic toxicity. In contrast, cationic lipid or polycationic–pDNA complexes induce a dose-dependent toxic response when delivered by aerosol into the lung or injected systemically. The response observed in CF patients that received aerosolized complex was transient and treatable symptomatically with analgesics. However, systemically delivered complex induces a much more severe reaction, especially at higher doses, and no clinical use with this delivery route can be foreseen until this toxicity is resolved. Less toxic vectors and formulations, which include CpG-depleted vectors and smaller nonaggregating complexes, are under development. But for now, any proposed clinical application should consider the possible consequences of the innate immune reaction to current synthetic vector systems.

Gene transfer efficiency continues to be the paramount limitation of current synthetic vectors. The many published preclinical results demonstrating some degree of efficacy are generally using delivery methods or doses that would not be clinically acceptable, and most studies have been in small animals such as mice, where the levels of expression observed are often not translatable to larger animals and humans. Therefore, generating therapeutic levels of a protein to replace that which is absent in a given inherited genetic disease is probably unrealistic with the current technology. More feasible is expressing modulating proteins, such as cytokines to inhibit inflammation, tumor-specific antigens to induce an immune response, or growth factors to promote angiogenesis or cell proliferation. These approaches, as well as related strategies in which synthetic gene transfer serves as an adjunct therapy to existing therapies, may be more appropriate and have greater chances of clinical success.

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41

Regulatory Aspects of Gene Therapy

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I. U.S. OVERSIGHT OF GENE THERAPY

A. Food and Drug Administration Regulatory Authority

Since the beginning of recorded history, societies have been concerned about the purity of the food and drink available to the public. Regulation of food in the United States dates back to early colonial times. Federal controls over the drug supply started in 1848 when the state of California passed a pure food and drink law. In 1902, Congress made appropriations to establish food standards; in the same year, the Biologics Control Act was passed to license and regulate interstate sale of serum, vaccines, and other biologics used to prevent or treat disease in humans. The 1902 act established federal inspection of licensed facilities, prohibition of false labeling, and the concept of a dating period during which a biological product would be medically used. In 1906, the Food and Drug Act passed Congress, prohibiting interstate commerce of misbranded and adulterated foods, drinks, and drugs. In 1938, the Federal Food, Drug, and Cosmetic (FD&C) Act was enacted, making the 1906 Food and Drug Act obsolete. The FD&C Act extended controls to cosmetics and therapeutic devices, and also required predistribution clearance of new drugs based on safety. In addition, this act authorized standards of identity and quality for foods and drugs, as well as authorized factory inspections. In 1944, the Public Health Service Act (PHSA) was established and consolidated the major rule-making authority for biological products under Sections 351 and 352. The PHSA requires that the product and the establishment where the product is manufactured meet standards to ensure continued safety, purity, and potency of the biologic.

Today the primary mission of the Food and Drug Administration (FDA) is to safeguard and promote the public health by promptly and efficiently reviewing clinical research and taking appropriate action on the marketing of regulated products. This is accomplished by upholding established regulatory principles, such as quality control, sound scientific rationale, and risk–benefit assessment. During the regulatory process, it is important that these principles be applied in a way that will encourage early product development and not inhibit development of new clinical approaches. This is particularly challenging for the field of gene therapy, where some of the risks are clearly undefined, although the potential benefit of these therapies may be great. To apply these principles, the Center for Biologics Evaluation and Research (CBER) takes a comprehensive approach to the regulation of biological products. This approach involves scientific review of submissions, a strong research program to support the regulatory process, surveillance, policy development based on sound science, and compliance to ensure that the manufacture of biological products and the conduct of clinical trials are performed in accordance with the regulations and statues set forth to safeguard subjects enrolled in clinical trials.

CBER is 1 of 5 centers comprising the FDA and is responsible for the regulation of biological products. The authority to regulate biologics is mandated by both the FD&C Act and the PHSA. These acts outline binding practices for the agency and the sponsor. The FD&C Act provides the legal interpretation that a ''biologic product'' is also a ''drug,'' and section 351 of the PHSA makes provisions for the regulation of biological products through licensure of the product and the establishment in which the product is manufactured. Under the PHSA, a biologic is defined as ''a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, analogous product, or arsphenamine or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment or cure of a disease or condition of human beings.''

Regulations pertaining to conduct of clinical investigations using biological products are outlined in title 21 of the Code of Federal Regulations (CFR). Regulations are interpretations of the laws that provide rules for daily business and are binding like laws. The regulations covered under 21 CFR 312 specify requirements necessary for submission of investigational new drug (IND) applications, whereas standards for licensure of a biological product are described under 21 CFR 610. The FDA's regulatory authority in the somatic cell and gene therapy area was established in the *Federal Register* dated October 14, 1993, entitled ''Application of Current Statutory Authorities to Human Somatic Cell Therapy Products and Gene Therapy Products.'' This document establishes that somatic cell and gene therapy products are biological products, and as such are subject to the licensing provisions of the PHSA.

Besides the statutes and regulations by which the FDA governs its day-to-day interactions with industry and academia, the agency also issues guidance documents that are not binding but rather describe CBER's policy and regulatory approach to specific product areas. Examples of documents that present recommendations, and provide relevant guidance to somatic cell and gene therapy products, are provided in section V.

B. Status of Gene Therapy in the United States

As provided in the document, ''Application of Current Statutory Authorities to Human Somatic Cell Therapy Products and Gene Therapy Products,'' human gene therapy is defined as a medical intervention based on the administration of genetic material in order to modify or manipulate the expression of a gene product or to alter the biological properties of living cells. Cells may be modified ex vivo for subsequent administration or altered in vivo by gene therapy products given directly to the subject. Examples that fall under this definition include, but are not limited to, autologous bone marrow stem cells modified with a viral vector, intramuscular injection of a plasmid DNA vector, and direct injection of a viral vector for treatment of hemophilia. Since submission of the first human gene therapy IND in 1989, 317 human gene therapy INDs have been submitted to CBER for review (through September 2002). The review of gene therapy INDs was the responsibility of the Office of Therapeutics Research and Review until September 30, 2002. Effective October 1, 2002, the review of gene therapy INDs is coordinated through the newly created Office of Cellular, Tissues, and Gene Therapies within CBER.

Currently, there are 197 active gene therapy INDs at CBER (Fig. 1). Although more than one-half of the INDs submitted have involved ex vivo modification of cells using gene therapy vectors, since 1995 the number of INDs involving direct administration of gene therapy vectors has been increasing. Currently, almost 48% of all INDs use direct vector administration. Overall, almost 90% of INDs submitted have involved expression or replacement of a gene with therapeutic intent and comprise indications as broad as cancer, HIV, cystic fibrosis, hemophilia, peripheral and arterial vascular disease, arthritis, and many rare diseases (i.e., x-linked SCID, Fanconi anemia). In approximately 26% of the INDs, nonviral plasmid vectors were used for gene delivery. Of the viral vector systems used, more than one-half of the INDs submitted have used retroviral vectors, about one-quarter adenoviral vectors, and a small number have used adeno-associated, herpes and vaccina viral vector systems.

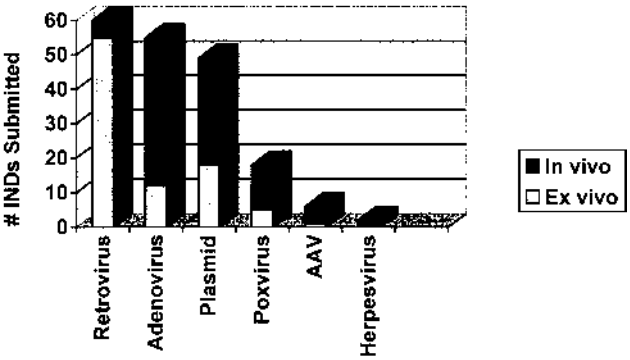

Figure 1 Schematic of currently active INDs showing the types and number of vectors being used in U.S. gene therapy clinical trials.

For the area of gene therapy, CBER has applied a unique regulatory approach that has included an element of public process that is not typically used to regulate other products at the FDA. The goal of this public interaction has been to increase the community's understanding of the CBER review process and requirements, to allow deliberation of ethical and social issues that surround the area of gene therapy, to receive input into CBER gene therapy policy development, and to provide accurate information to the public about the progress of gene therapy clinical trials. Much of this process has been facilitated by the National Institutes of Health (NIH) Office of Biotechnology Activities (OBA), through quarterly meetings of the Recombinant DNA Advisory Committee (RAC). The FDA also convenes its Biological Response Modifiers Advisory Committee to discuss relevant issues regarding the safe use of gene therapy products and conduct of clinical trails. In addition, the FDA routinely takes part in forums with industry, trade groups, academia, and the public in order to foster public understanding in the area of gene therapy, which is essential for continued progress of the field.

C. FDA/RAC Oversight

Unlike other areas under clinical investigation that are regulated by the FDA, gene therapy clinical investigations are subject to the scrutiny of 2 federal agencies within the Department of Health and Human Services, the FDA, and the NIH OBA. Oversight of human gene therapy clinical trials at the NIH involves a public process of review and discussion, conducted

by the RAC, that ensures public awareness of clinical trial registration and follow-up. The RAC meets quarterly for public discussion of proposals that are deemed novel. In contrast, FDA review of gene therapy INDs is confidential and conducted by agency reviewers on an ongoing basis.

The emphasis of the review by each group is complimentary. Both the NIH and the FDA deliberate preclinical and clinical issues; however, the RAC's responsibilities extend beyond safety and efficacy to the consideration of the ethical, legal, and social implications of such research. The FDA provides careful and thorough review of product manufacturing related to product safety, purity, potency, and identity, whereas the RAC does not consider these manufacturing issues due to the proprietary nature of such information. Currently, information regarding human gene therapy clinical trials must be submitted to both the FDA and the NIH.

1. RAC History

The RAC was established in 1975 as a result of public concern over the potential risks of the new field of recombinant DNA (rDNA) research. Scientists worldwide had voluntarily halted their research and met in Asilomar, California, to debate the future of the use of rDNA technology. The RAC evolved from these debates and met for the first time just after the Asilomar meeting. The RAC mission was to advise the NIH Director and to review in public each experiment involving recombinant DNA research. Subsequently, NIH established the OBA (previously called the Office of Recombinant DNA Activities), to provide administrative support to the RAC. Over the first few meetings, the RAC set minimum standards for biological and physical containment of rDNA molecules. This was accomplished through public debate and with input from scientists and lay representatives, including ethicists and economists. In 1976, as a result of these discussions, the NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines) were published in the *Federal Register*. The NIH Guidelines provided for submission and review of rDNA experiments by the RAC and also provided for the element of public debate of rDNA research. The NIH Guidelines are not regulations, but establish their authority through the NIH funding process. Investigators receiving NIH funding, or who are affiliated with an institution that has NIH funding, must comply with the NIH Guidelines. In addition, they ask for voluntary compliance by non–NIH-funded investigators. This process has provided a precedent for the public discussion and consideration of gene therapy clinical trials conducted today.

In 1982, in response to the report of the President's Commission entitled ''Splicing Life: Social and Ethical Issues of Genetic Engineering with Human Beings,'' the Human Gene Therapy Subcommittee (HGTS) to the RAC was established to review the application of rDNA technology to human gene therapies. The first human gene therapy protocol was approved after public discussion by this committee and separately by the FDA in 1990. Over time, as the public concern over rDNA experiments subsided and the interest in gene therapy increased, the HGTS subcommittee merged with the full RAC and the combined group discussed and approved each gene therapy protocol prior to its initiation. In 1994, an accelerated review process was adopted for certain categories of clinical trails that had been routinely reviewed by the RAC and determined not to represent significant risk to human health and the environment. Under this mechanism, such protocols were subject only to written review by several RAC members, and OBA approval, outside the quarterly meetings.

The RAC is currently comprised of 21 members from the disciplines of science, medicine, law, and ethics. They meet quarterly for the public review and discussion of novel gene therapy protocols. However, they no longer approve or disapprove protocols. Currently, their function is purely to provide a platform for public discussion of novel issues involved in gene therapy clinical trials. In addition, the RAC sponsors the Gene Therapy Policy Conferences (GTPC), which provide a mechanism for in-depth discussion of relevant gene therapy issues. For this forum, a panel of experts is convened with the goal of reaching consensus and developing guidance in a particular area. GTPC discussions have focused on topics such as genetic enhancement, use of lentiviral vectors, prenatal gene therapy, and adeno-associated virus (AAV) vectors.

2. Dual Submissions

As illustrated in Fig. 2, there is a parallel path of submissions required prior to initiation of a gene therapy clinical trial. Sponsors of a gene therapy clinical trial must submit an IND application to the CBER, FDA for review under a 30-day review cycle and may not proceed until the IND is found to be acceptable. In addition, the FDA requires that a sponsor receive the approval of the institutional review boards (IRBs) and institutional biosafety committees (IBCs) affiliated with the institutions at which the trail will be conducted. Concurrently or prior to submission of an IND to the FDA, investigators conducting the clinical trial must also submit their protocol and information specified in Appendix M of the NIH Guidelines to the NIH OBA for review by the RAC. Based on recent changes to the NIH Guidelines for Research Involving Recombinant DNA Molecules (May 2002), all investigators/sponsors who receive or who are sponsored by an entity that receives NIH support for recombinant DNA research must submit their clinical protocol to the RAC for review. Failure to follow the NIH Guidelines can result in withdrawal of NIH funding to the investigator and any supporting institutions. Although an investigator may submit the protocol to the FDA and the IRB prior to submittal to the RAC, review by the RAC is required before final IBC approval, so the committee can be informed of the RAC recommendations before making its final determination. The OBA submission must be received no less than 8 weeks prior to the next scheduled RAC meeting. A gene therapy clinical trial will be judged as exempt from, or in need of, full RAC/public discussion based on the following factors: novelty of the vector or gene delivery system, special disease concerns, unique applications of the gene therapy research or important social or ethical issues raised by the proposed research. Recommendations and comments resulting

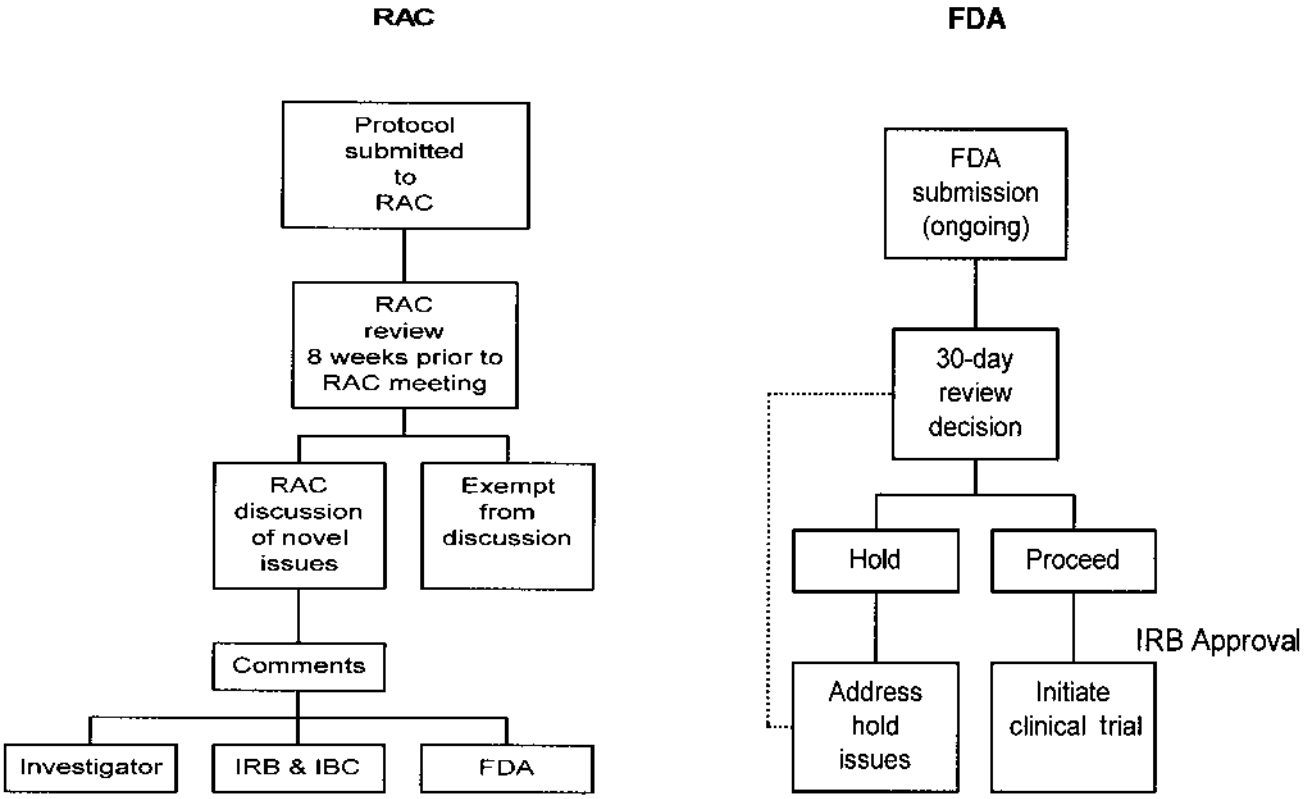

Figure 2 Overview of parallel processes for submission of documentation related to initiation of human gene therapy clinical trials to the RAC and the FDA.

from discussion at the RAC meetings are forwarded to the investigator, the IRB, IBC, and FDA.

Due to timing and regulatory requirements, there are situations where the FDA decision to allow a clinical trial to proceed must be made before public discussion can occur. In this situation the investigator/sponsor is reminded by the FDA of the need to comply with NIH Guidelines regarding DNA recombinant research and that enrollment of subjects into the clinical trial may not begin until review and, if selected, public discussion of the protocol by the RAC has taken place. In general, public discussion of gene therapy clinical trials has been highly beneficial because it allows for consideration of societal and ethical issues surrounding the field of gene therapy and ensures the continued public acceptance and progression of the field.

II. THE IND PROCESS

As mandated under section 505 of the FD&C Act and section 351 of the PHSA, it is illegal to sell or distribute any biologic unless it is licensed or under an Investigational New Drug Exemption (21 CFR 312.2). Submission of an IND to provide for this exemption allows clinical investigation of the product to proceed in order to determine safety, dosage, and effectiveness. This investigational process is usually divided into 3 phases, with each phase providing the next step in support of product licensure.

A. Definition of IND Phases

As illustrated in Fig. 3, product development begins with the pre-IND stage and progresses through the investigational or IND stage (phases I–III), where data are obtained to support product licensure. This is followed by the postlicensing stage (phase IV), during which further studies are often performed. An IND may be submitted for 1 or more phases of an investigation, although, in general, the phases of a clinical study are conducted sequentially.

A phase I trial includes the initial introduction of an investigational new drug into humans. The primary focus of a phase I study is to monitor product safety in a specific patient population, although it should be noted that assessment of product safety remains a primary issue throughout product development. Phase I studies should be designed to determine the metabolic and pharmacological actions of the drug in humans and the side effects associated with increasing doses, and, if

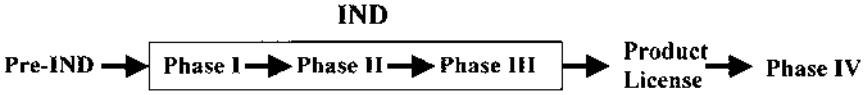

Figure 3 Phases of product development and approval.

possible, to gain early evidence of product effectiveness. During the phase I study, the investigator should focus on obtaining sufficient information about the drug's pharmacokinetics and pharmacological effects that would permit for the design of well-controlled, scientifically valid, phase II studies. In a phase I study, the product may also be assessed for structure–activity relationships, as well as the mechanism of action in humans. The total number of subjects included in a phase I gene therapy study varies but is typically between 10 and 40.

Phase II of an IND should include controlled clinical studies conducted to evaluate the effectiveness and dose ranging of the product for a particular indication in patients with the disease or condition under study. These studies should be designed to determine common short-term side effects and risks associated with the biological product. Phase II studies are typically well-controlled, closely monitored, and conducted in a relatively small number of patients, usually involving no more that several hundred patients.

Phase III studies are expanded well-controlled, randomized and, when possible, blinded studies that are performed after preliminary data for the effectiveness of the product have been obtained. These studies are intended to gather further information about product effectiveness and safety that is needed to evaluate the overall benefit–risk relationship of the product and generally serve as the pivotal efficacy study to support licensure. Phase III studies usually include from several hundred to several thousand patients, depending on the clinical indication and patient population.

Clinical evaluation of a biological product rarely ends with issuance of a biologics license. Phase IV, or the postmarketing stage, refers to the ongoing period of development after the product is licensed. Examples of postmarketing studies include clinical studies to extend claims or usage for the addition of a new patient population or indication, studies to demonstrate product comparability after manufacturing changes, and studies to validate surrogate clinical endpoints that are required in cases of expedited review and accelerated approval.

The FDA's primary objective when reviewing all phases of an IND is to assure the safety and rights of the patient, and later in phases II and III, to help assure that the validity and quality of the scientific data used to evaluate the product is adequate to assess product safety and efficacy

B. Pre-IND Phase

An IND application and the application process itself can be bewildering to novice sponsors. Therefore, before IND submission, CBER encourages an early interaction, in the form of a pre-IND meeting, to discuss preclinical animal testing, product development, and clinical trial plans. Although the central focus of the pre-IND meeting is to define what is needed to support the IND submission, another important goal is to create a dialogue between CBER and the sponsor/investigator that can be maintained throughout the process of product development. The pre-IND meeting is arranged at the request of the sponsor; however, before CBER can grant a pre-IND meeting, the sponsor will need to prepare a meeting package. Although the investigational plan does not have to be in its final form, the sponsor should be prepared to briefly describe all aspects of the proposed clinical study in the meeting package. This should encompass a description of the biological product, product manufacturing and testing schemes, established preclinical data, plans for additional preclinical studies, the proposed clinical protocol, and the general proof of concept behind the proposal. Most important, the pre-IND meeting should be used as a mechanism for focusing on unresolved issues relating to preclinical studies, clinical studies, or product development. It is recommended that questions be submitted with the pre-IND package addressing any concerns or potential problems with the investigational plan that would require guidance and/or discussion with the agency before submission of the IND. Past experience has proven that early identification and resolution of these issues will ultimately enhance and accelerate the product development process.

C. The IND Phase

Before submitting an IND, the sponsor must have generated enough preclinical data to ensure the safety of the proposed clinical trial. Preclinical data can be generated using either in vivo animal studies or in vitro studies to assess the product's activity, efficacy, pharmacology, pharmacokinetics, and toxicity. In addition, preclinical studies should be designed to identify potential target organs of toxicity and provide data to support clinical use of the product, such as safe starting dose, route of administration, and dosing regimen. Information to support the scientific rationale behind the proposal should also be provided. Issues specific for gene therapy that can be addressed with preclinical studies include biodistribution or trafficking of vectors, level and persistence of gene expression, and germline alteration. The sponsor should also have a well-developed and controlled product-manufacturing scheme, and have collected data regarding product characterization and consistency to support the manufacturing process.

This data should also be used to support proposed specifications for product quality control and release.

Once these issues have been addressed, the next step is the preparation of the IND submission. For assistance in preparation of an adequate and complete IND, the sponsor should contact the Office of Communication, Training and Manufacturers Assistance to request an IND submission package. The package contains forms to be submitted, copies of the IND regulations, informed consent and IRB regulations, information pertaining to GLP/GMP, and the essentials required for conducting adequate and well-controlled clinical trials. An IND submission package can also be obtained at the FDA website (www.fda.gov/cber/ind/ind.htm).

1. The IND Submission

The content and format of an IND submission is specified in 21 CFR under part 312.23. This part lists, in order, the items that a sponsor (person who takes responsibility for and conducts a clinical investigation) should submit in the IND (Fig. 4). The IND should be submitted to CBER in triplicate and, upon receipt the sponsor of a human gene therapy, IND will be issued an acknowledgment letter containing the date of receipt, the assigned IND number, and a reminder of their responsibility for submission to NIH OBA according to Appendix M of the NIH Guidelines. The IND receipt date begins the official review clock and the IND review will take place over the next 30 calendar days. INDs automatically become effective 30 days after receipt unless the FDA notifies the sponsor that the IND is subject to clinical hold. In the CBER, where human gene therapy INDs are reviewed, the IND review team is comprised of a product reviewer, a pharmacology/toxicology reviewer, a clinical reviewer, and a regulatory project manager who handles administrative aspects of the IND review. The product reviewer is responsible for coordinating the review team and ensuring consistency within a product area. Consult reviewers are used on an ad hoc basis, depending on the product and its application, and could come from other offices in CBER or other centers within the agency, depending on the required expertise.

At any point in the review process, a reviewer may call the sponsor to request additional information or to discuss deficiencies in the IND. After each reviewer has completed their review, the team meets to discuss the file and make a final decision on the status of the application. The IND may also be discussed at office-level meetings for further input. The review decision is communicated to the sponsor by phone within the 30-day period, and this is followed by a letter giving the details of hold issues, review comments, or requests for further information. The clinical trial may either proceed or be placed on clinical hold. Phase I INDs may be placed on clinical hold, as covered under 21 CFR 312.42(b), if human subjects are exposed to unreasonable and significant risk of illness or injury; the IND does not contain sufficient information to allow adequate assessment of the risk (21 CFR 312.23); the information in the investigators brochure is misleading, erroneous, or materially incomplete; or the clinical investigators are not qualified to conduct the study. In addition, for investigational studies of drugs intended to treat a life-threatening disease or condition that affects both men and women, phase I INDs may be placed on clinical hold if men or women of reproductive potential are excluded from the clinical trial because of reproductive risk, or the potential of reproductive risk, due to the reproductive or developmental toxicity associated with the drug. Phases II and III INDs may be put on clinical hold if any of the conditions cited above apply or if the protocol design is deficient to meet the objectives of the proposal. To proceed with the clinical study, the sponsor must correct the deficiencies identified during the review and submit the additional information or data in an amendment to the IND. Once the amendment has been submitted and reviewed by the FDA, the sponsor will be notified by phone that the clinical trial may proceed, followed by a written letter.

a. Annual Reports. An annual report describing the progress of the investigation should be submitted to the IND within 60 days of the anniversary date that the IND went into effect. 21 CFR part 312.33 should be consulted for specific details, but in general the annual report should provide information on the status of each study in progress or completed during the previous year. To this effect, the annual report should include an update on the following: the title of study(s), the number of subjects enrolled and their status, serious adverse experiences observed, summary of available study results if the study is completed, information relevant to understanding of the drug's action, results of additional preclinical studies performed during the year, manufacturing changes, and an investigational plan for the coming year. The annual report should also provide results of product characterization and lot release testing for all lots of product produced during the year under report. Annual reports for human gene therapy trials should include additional information relevant to gene therapy vectors, such as assessment of evidence of gene expression, biological activity, immune response, evidence of gonadal distribution, results from long-term patient follow-up, and status of requests for autopsy. The annual report should also contain an update on information requested in the March 6, 2000 gene therapy letter, posted on the CBER website (www.fda.gov/cber/ind/ind.htm).

2. Master File Submission

Another mechanism available for submission of information to the FDA is the Master File (MF). The procedures for submitting a MF are outlined in part 312.33 of 21 CFR. In contrast to the IND, which contains manufacturing, preclinical, and clinical information, the MF could contain product manufacturing, preclinical, clinical, or facilities information only. Submission of an MF allows the MF holder to incorporate the information by reference when the MF holder submits an IND. The MF holder may also authorize other sponsors to rely on the information to support an IND submission without disclosing the information to the sponsors. There are 4 types of Master File submissions, of which type II is the one most commonly used for biological product development. Information submitted in a type II Master File can include information on drug substance or drug product specifically regarding product

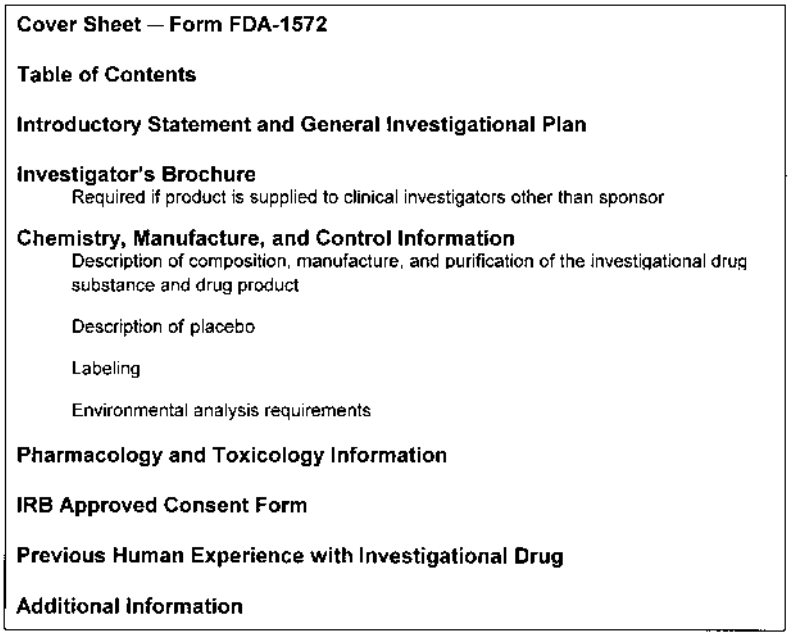

Figure 4 Content and format of the IND submission as specified in 21CFR 312.23.

manufacturing and purification schemes, SOPs, lot release protocols, tests and specifications, descriptions of tissue culture media components, and other proprietary information needed to support an IND application. When granting written permission for a cross-reference, a copy of the cross-reference letter should be filed with the MF and the IND it is supporting, and should identify by name, reference number, and volume and page number the exact information that each IND sponsor is authorized to incorporate. For example, in the case where retroviral vector supernatant is provided to an IND holder by a manufacturer and the method of manufacture is proprietary, the manufacturer could submit an MF documenting retroviral vector production and testing in support of the IND for review by CBER staff. As product lots are produced, lot release data should be submitted to the product MF as well as to the cross-referenced IND for CBER review.

MFs are often reviewed only in the context of an IND application. CBER will comment on the contents of the MF and ask for clarifying information in order to more adequately review an IND that cross-references the MF; however, a MF is neither approved or disapproved. Importantly, the MF has to contain complete information to support the decision to allow the associated IND to proceed.

III. REVIEW CONSIDERATIONS: EARLY PHASES

A. Stepwise Approach

Product characterization and quality control of gene therapy products includes issues and concerns common to most biological products, such as demonstration of safety, development of methods for assessment of potency, determination of identity and purity, and product stability. In addition, development of specifications for each parameter is an important part of product development and characterization, with specifications being established and tightened over time as data become available. Another aspect common among biologics is control and regulation of not only the product, but also each step of the manufacturing process. Adherence to this approach, which is encompassed under current good manufacturing practices (cGMPs), provides for quality and safety throughout the process and will lead to consistency of product lots.

In the area of gene therapy more than in other areas, a flexible approach to regulatory requirements has been attempted in order to find a balance between ensuring patient safety and fostering development of the field. To facilitate this process, a stepwise approach to product characterization and compliance with cGMP has been adopted. This approach involves a progressive scale of requirements for product characterization and compliance with cGMPs, which increase as the study moves from phase I toward phase III (Fig. 5). The first step in this approach is to provide preclinical toxicity data obtained from an appropriate animal model. The next step, which occurs during phase I of the clinical trial but is required throughout all product development, is the demonstration of product safety. As the clinical trail proceeds, so should the characterization of the product, such that by phase III the product is fully characterized with regards to safety, purity, identity, and potency. In addition, by phase III the product should be produced under full compliance with cGMP regulations. Requirements for licensure of a biological prod-

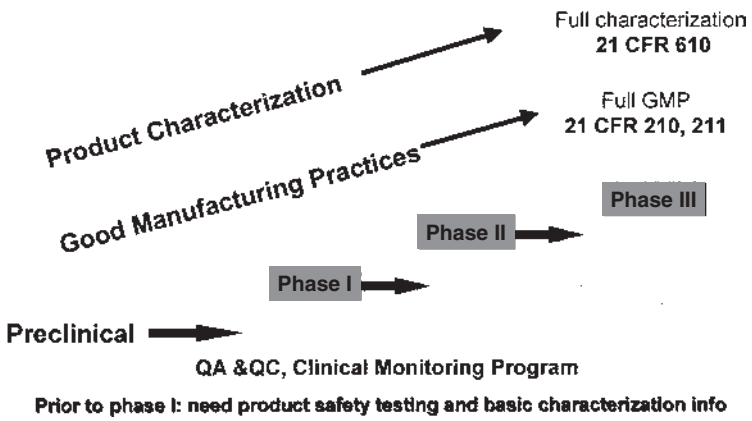

Figure 5 The stepwise approach.

uct, product standards, and cGMPs are specified in parts 610, 210, and 211 of section 21 of the CFR. The stepwise approach applies to each aspect of gene therapy product manufacture, including vector development, establishment and characterization of cell banks, product manufacture and characterization, establishment of specifications for product release, and control of reagents used during manufacture (ancillary products). It is equally important that a quality assurance/quality control (QA/QC) program, which is separate from manufacturing, be in place at the earliest phase of product manufacture. The QA/QC unit should be responsible for ensuring the quality of the product and for product release for clinical use. The role of the QA/QC unit is described in part 211 of section 21 of the CFR. Control of each aspect is important to assure product consistency and safety, and is the focus of the Chemistry, Manufacturing, and Control (CMC) review.

B. CMC Review

Each step of product development, characterization, manufacture, and control should be described in the IND under the CMC section. Sufficient information is required in this section to assure the proper identification, quality, and safety of the investigational drug. For phase I trials, data must be submitted to establish product safety. In addition, information regarding product characterization should include a description of the product's physical, chemical, or biological characteristics; the method of manufacture; analytical methods used during manufacture and testing; and initial product specifications. The emphasis of the review of an IND submission is on data; therefore, in all cases data, as opposed to conceptual information, should be provided in support of the IND. Valid scientific principles should be applied throughout product development with regard to product safety, characterization, and quality control of the manufacturing process. To ensure product quality, there should also be proper testing in place for all the components used during product manufacture. During the review process, a case-by-case approach is applied in order to ensure that requirements and recommendations are satisfied in a manner appropriate to a particular product and manufacturing method.

An early step in the development of a gene therapy product is construction of the initial gene therapy vector. The information supplied to the IND regarding the vector should include an explanation of the derivation of the vector, a description of the vector components and their sources, including the gene insert and regulatory elements, and a vector diagram. Currently, the FDA requires that all gene therapy vectors less than 40 kb be fully sequenced prior to initiation of a phase I clinical trial. A fully annotated DNA sequence and sequence analysis report should be submitted, identifying all open reading frames (expected and unexpected) and genes encoded in the vector. The report should indicate whether there is sequence alignment between the vector and the expected viral and inserted DNA sequences, as identified by a search of a relevant current database. For vectors over 40 kb, sequence analysis of the gene insert, flanking regions (at least 500 base pairs either side), and any regions of the vector that have been modified should be submitted.

In general, the next component important for the manufacture of a gene therapy product is the cell. Cells used during production of a gene therapy vector can be prokaryotic or eukaryotic. Eukaryotic cells can be either autologous or allogeneic. Information that should be submitted in the CMC section regarding the producer cells includes the cell source, collection method, whether a mobilization protocol is used, and donor testing (if applicable). If a cell bank system is used to assure consistency of production, then the next step in product development is usually the establishment of a Master Cell Bank (MCB). MCB characterization includes testing to establish the properties and stability of the cells and is performed on a one-time basis. For the IND submission, information on the history of the cell line, as well as culture and storage conditions of the MCB, should be included. Testing should be performed to establish identity, which includes cellular

phenotype and genotype, cellular isoenzyme expression, and stability. If the cell line has been engineered to express exogenous genes, then tests should be performed to establish the presence and stability of the transgene. Safety testing should be performed to demonstrate that the MCB is sterile and free of mycoplasma, endotoxin, and adventitious agents (through in vitro and in vivo assays). Depending on the origin, MCBs should be tested for species-specific pathogens. For example, if the MCB is of human origin, then testing for human pathogens such as CMV, HIV-1 & 2, HTLV-1 & 2, EBV, B19, AAV, HBV, and HCV must be performed. Analytical methods used for testing should be summarized and initial specifications for qualification of the MCB established. The MCB is the first tier of a two-tier cell bank system; the second is the Working Cell Bank (WCB), which is derived from 1 or more vials of the MCB. The amount of information needed for characterization of the WCB is generally less extensive and includes testing for sterility, mycoplasma, adventitious agents by in vitro assay, and identity. Additional information on cell banking procedures and cell bank characterization can be found in the ''Points to Consider in the Characterization of Cell Lines to Produce Biologicals'' (1993).

Depending on the gene therapy vector, the next step in gene therapy product development may be the establishment of a Master Viral Bank (MVB). MVBs are generated by infecting or transducing the specific vector into cells grown from 1 or more vials of the WCB. As with MCBs, a second tier or Master Working Viral Bank (MWVB) can also be generated. Requirements for MCB/WCB testing and characterization would also apply to testing of the MVB/MWVB, with the addition of tests for the presence of RCV related to the vector.

All procedures used during production and purification of the gene therapy vector product should be included in the CMC section of the IND submission. Safety testing and characterization of gene therapy vectors should be performed on bulk production lots, before and after purification steps, and final product after formulation and fill, as appropriate for the particular assay. Safety testing should include tests for sterility, mycoplasma, endotoxin, general safety, and RCV, as well as tests for adventitious virus by in vitro assay. Product identity for a gene therapy vector product may be established through the physical or chemical characteristics of the product using in vitro, in vivo, or, when appropriate, molecular methods, but is not absolutely required at phase I. Purity of a gene therapy vector is typically established through determination of levels of residual materials such as cellular DNA, RNA, and protein; noninfectious virus; and ancillary products. With regard to product potency, for early phase trials the level of gene expression is acceptable as a measure of potency. However, before initiation of a phase III clinical trial, a potency assay that measures the relative biological activity of the product will need to be developed and in place. A suitable potency assay should be one that is quantitative in nature, but in addition, can also include a qualitative biological assay. As with testing of the MCB and MVB, analytical methods used should be summarized in the IND and initial specifications for qualification of the gene therapy vector product established. This summary is often presented in the form of a Certificate of Analysis.

To date, approximately 48% of gene therapy clinical trials have involved the genetic modification of autologous or allogeneic cells ex vivo. This has been accomplished using viral and nonviral vectors. Information regarding the process of ex vivo modification of cells should be documented in the IND submission as follows: a description of the source of cells, results of donor screening (if applicable), method of cell collection and processing, culture conditions, and the procedure for ex vivo modification of cells. Safety testing and characterization of the ex vivo gene-modified cells should include assessment of sterility, mycoplasma, endotoxin, identity, potency, and freedom from RCV (for retrovirus vectors). Analysis of phenotypic markers and confirmation of the integrity of the genetic insert may be used to confirm cell identity. Care should also be taken to assure patient specificity for autologous products using proper labeling and tracking systems. Additional parameters to be assessed include cell viability, transduction efficiency, longevity of gene expression, and the effects of irradiation or freeze/thaw on these parameters. In addition, the vector used for ex vivo modification should be tested as described above before use in the ex vivo modification of the cell product. Analytical methods used should be summarized in the IND and initial specifications for qualification of the ex vivo modified cells established.

For gene therapy vector products and ex vivo modified cell products, a stability program that assesses product safety, activity, and integrity should be established during early phases of investigation. The objectives of stability testing during early phases are to establish that the product is stable for the duration of the clinical trial and to collect information needed to develop a final formulation and dating period. The submission to the IND should include a brief description of the proposed stability study, the test methods to be used to monitor stability, and preliminary data, if available.

The stepwise approach provides flexibility to product manufacture, characterization, and testing during early phases of product development. However, testing and assay qualification should be performed in early phases to support data collection, development of specifications, and compliance with cGMP in preparation for phase III studies.

C. Reagents

Reagent are those components that are essential for cellular growth, differentiation, selection, purification, or other critical manufacturing steps, but that are not intended to be part of the final product. Examples include fetal bovine serum, trypsin, growth factors, cytokines, monoclonal antibodies, cell separation devices, medium, and medium components. These reagents can affect the safety, potency, and purity of the final product, especially with regard to the introduction of adventitious agents. Ideally, licensed or clinical grade reagents should be used for preparation of gene therapy products; however, these are often not readily available. Recommendations for the use of reagents that are not clinical grade during early

phases of product development involve establishing a qualification program and specifications for each reagent. The qualification program should include adequate characterization, including safety testing, functional analysis, and a demonstration of purity. The purity of a given reagent need not be 100%, but the purity profile between different lots of the reagent should remain consistent. The extent of reagent testing required will depend on the point at which the reagent is used in the manufacturing process and the biological system used for production of the reagent. For example, in the case of a growth factor or cytokine, if a monoclonal antibody is used during production or purification, then the reagent will have to be tested for adventitious agents and murine retrovirus. If there are known toxicities associated with the reagent, testing for residual levels of the reagent in the final product preparation should be performed. It should be noted that the use of reagents produced under full cGMP is recommended for phase III trials.

D. Gene Therapy-specific Safety Issues

As the development of gene therapy vectors for therapeutic gene delivery broadens and moves forward, the possibility of approved therapeutic products becomes more of a reality. However, regulatory approval and use of these therapeutic products in clinical trails requires a good safety profile. The use of gene therapy vectors carries with them certain safety issues that are specific to each vector type. Concerns specific to the safe use of gene therapy vectors include the generation of replication-competent virus (RCV), vector-associated toxicity (as opposed to toxicity related to the specific transgene), and the risk of inadvertent modification of a recipient's germline. Because of their potential effect on the patient, the public health, and future generations, these issues need to be addressed during development of a gene therapy product and should be documented in the IND. Because RCV can arise from recombination events during manufacture, tests designed to detect RCV should be performed at multiple stages of manufacture and for each final lot.

Testing for replication-competent retrovirus (RCR) in the production of retroviral vectors needs to be performed at multiple points in production, including MVB, WVB, vector supernatants, end of production cells, and ex vivo modified cells. A permissive cell line such as *Mus dunni* should be used to test cells producing vector containing the amphotropic murine leukemia virus envelope. If an ecotropic packaging cell line is used, then an ecotropic retroviral assay, for detection of low-level viral contamination, will need to be performed.

Retroviral vector supernatant also must be tested by amplification on a permissive cell line such as *Mus dunni*, followed by detection in an appropriate indicator cell assay (PG-4 S+L-) . When the final product is ex vivo transduced cells, RCR testing is recommended if cells are maintained in culture for 4 or more days after transduction. If ex vivo transduced cells are cultured for less than 4 days, then archiving of cells is recommended in place of active RCR testing. Further information regarding RCR testing is contained in the CBER guidance ''Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors, 10/18/2000''

In studies using adenoviral vectors, MVBs and WVBs, as well as the final vector product, must be tested for replication-competent adenovirus, (RCA). The FDA recommends that the level of RCA in the final product be less than 1 in 3×10^{10} viral particles.

Adenoviral vectors pose a specific safety risk because toxicity has been observed when these vectors are administered at high doses. Current evidence suggests that a portion of the observed toxicity is due to the vector particle itself, and is not related to the actual infection of the cells by the vector. As a result, CBER requires that adenoviral vector doses be based on the actual particle count, as opposed to the infectious titer, which is commonly the unit for quantifying viruses. In addition, the agency currently asks that all sponsors of INDs using adenoviral vectors report the ratio of viral particle to infectious unit for each clinical lot of vector. The current recommended limit for this ratio is set at ≤ 30.

After the death in 1999 of a subject enrolled in an adenoviral gene therapy clinical trial, the NIH OBA established the adenoviral Safety and Toxicity Working Group, whose mission was to conduct an indepth review and evaluation of safety and toxicity data generated from adenovirus gene therapy clinical trials. One outcome of this working group was a recommendation that a qualitative and quantitative adenovirus standard be developed. In response to this recommendation, the FDA, the Williamsburg BioProcessing Foundation, and members from industry and academia formed a working group to develop and characterize an adenoviral reference material (ARM). The ARM will be used by manufacturers of adenoviral vectors to standardize viral particle and titer measurements. In addition, use of the ARM will provide a means of analyzing the safety and efficacy of adenoviral vectors produced by different manufactures and allow for comparison between different clinical trials.

Safety testing goes beyond testing of the product and should include monitoring programs for all subjects who are administered gene therapy vectors. Currently, the FDA is developing a long-term patient follow-up program that will monitor all subjects receiving gene therapy products for unanticipated adverse events that manifest themselves long after administration of the gene therapy product and after the observation period for acute toxicity, as well as product efficacy, has ended.

For in vivo administered gene therapy vectors, data must be submitted demonstrating the extent to which a vector is able to disseminate out of the injection site and distribute to the gonads. These data will provide the information required to assess the risk of inadvertent gene transfer to germ cells, which may result in genetic changes in subsequent progeny. In general, these data should be obtained during the course of product development and provided to the agency for comment and review. However, in cases where a novel vector,

route of administration, or vector delivery system is proposed, preclinical studies may be required before initiation of the phase I study. Biodistribution data may be obtained from studies in animals, from analysis of clinical samples, or from a combination of preclinical and clinical sample analyses. Clinical data should be derived from peripheral blood cells and semen samples taken during the treatment and follow-up periods for the clinical trial and from gonadal tissues obtained at autopsy from consenting patients. The agency should be updated of the status of these studies at least at the time of each annual report in order to guide further product development and track any potential adverse events. A statement explaining the risk for genetic alteration of sperm or eggs and possible outcome for a fetus and future child, which could occur as a result of study participation, should be included in the informed consent document. This statement should clearly explain the status of biodistribution studies to assess gonadal distribution for each particular clinical trial and the fact that the likelihood of an adverse outcome is currently unknown. In addition, the consent form should advise study participants to practice birth control for a suitable period of time.

IV. REVIEW CONSIDERATIONS: LATER PHASES

The previous sections have provided an overview of the review process and CBER's expectations for product development as the clinical investigation proceeds through early phases. By phase III, CBER recommends adherence to product standards and methods specified in the regulations and also that products are manufactured under full cGMP compliance. To better understand CBER's requirements for product licensure and establishment standards, a sponsor should be familiar with practices that are governed by law and specified in parts 610, 210, and 211 of section 21 of the CFR. Part 610 provides information pertaining to product release requirements and general provisions for licensure and, in some cases, will provide information on specific assay methodology; parts 210 and 211 explain the cGMPs.

Although the regulations by their nature proscribe requirements, there is a level of flexibility built into the regulations that allows for modification of required test methods or manufacturing processes. The provision for test method modification is found under 21 CFR 610.9, entitled ''Equivalent Methods and Processes.'' This provision is especially useful for gene therapy products, where conditions of the manufacturing process and many times the product itself make it difficult to perform standard assays. To apply this provision, a sponsor should provide supporting evidence for why a specific method is not ideal for the gene therapy application and present data, with appropriate controls, to demonstrate that the modified method will give equal or greater assurances of the safety, purity, potency, and effectiveness of the biological product compared with the specified method or process. Data to support the modification can be accumulated during the early IND phases and then validated in support of its use as an established method prior to licensure.

For a product to meet the requirements for licensure, it must be fully characterized prior to submission of the license application with regard to safety, purity, potency, and identity. Each of these aspects will be discussed in the context of requirements for initiation of a phase III trial and product licensure.

Demonstration of product safety requires the implementation of specific tests, which measure sterility, mycoplasma, endotoxin, and general safety of the product. Product safety also includes demonstration that the product is free from adventitious virus, which to support the phase III study and product licensure includes in vitro and in vivo adventitious virus testing of the MCB and MVB, and when appropriate testing for RCV. Testing for general safety is not as rigidly adhered to during phase I and II studies, but by phase III the required standards and methods specified in 21 CFR 610.11 should be in place. An exception is made for therapeutic DNA plasmid products (21CFR 610.10) and cellular therapy products (FR Notice, April 20, 1998), which have been exempted from this testing.

Potency is defined as the specific ability or capacity of the product to effect a given result. For phase III, tests for potency should consist of quantitative in vitro or in vivo tests. A qualitative assay may be used as an additional demonstration of product potency. A potency assay that reflects the relevant biological function of the product, as opposed to an assay that just measures the level of gene product expression, should be optimized and qualified by phase III and validated prior to licensure. A valid potency assay is essential in order to interpret clinical trail results, as well as to ensure lot to lot consistency and quality of the product after licensure.

Product identity can be demonstrated through the use of an assay or assays that are specific for each product, in a manner that will adequately identify it as the designated product and distinguish it from any other product being processed in the same facility. At phase I, a test for identity is not required but it should be in place by phase II, so that data can be collected, specifications determined, and the assay validated by licensure.

Product purity is defined as freedom from extraneous material except that which is unavoidable in the manufacturing process. Testing for purity of a gene therapy product could involve assays for residual protein, DNA, RNA, solvents used during production and purification, or ancillary products used during manufacture such as cytokines, antibodies, or serum. As with all the previously mentioned testing, a quantitative assay for purity should be in place by phase III, with development of the assay being initiated much earlier in the investigational trial.

To complete product characterization in support of phase III and product licensure, a stability testing program that includes, but is not limited to, assays that measure product integrity, potency, sterility and, in the case of an ex vivo modified product, viability is needed. A stability protocol for study of both the bulk and final drug products, as well as all MCBs

and MVBs, should be defined so that stability data generated during phase III will be appropriate to support licensure. In general, the stability program should be initiated at phase I, so that by phase II the objective of obtaining real time data to support stability of the investigational formulation can be met. For phase III, data collected should be used to support the proposed expiration dating period of the final drug product, as well as the container and closure system. In addition, stability testing of any product intermediates should be in place to support the validation of the duration and conditions of storage of the bulk product.

A. cGMP

The second element to which the stepwise approach applies is the cGMPs. cGMP is defined as a set of current, scientifically sound methods, practices or principles that are implemented and documented during product development and production to ensure consistent manufacture of safe, pure, and potent products. Some major elements of cGMP include detailed record keeping, development of written procedures or standard operating procedures institution of QC/QA programs and assays, and equipment and process validation. cGMP also requires a program be in place for the certification and training of personnel and for environmental monitoring. The approach to cGMP is also flexible at early stages of product development. cGMPs play an important role in control and regulation not only of the product, but also of all steps of the manufacturing process. Adherence to cGMPs provides for quality and safety throughout the process and will lead to reproducible and consistent performance of product lots. It should be noted that cGMPs apply to the manufacturing process and to the facilities. The differences between phase I and phase III requirements are the degree to which each element of cGMP is implemented. As product development proceeds toward phase III, so should the validation of the conditions under which the product is manufactured, controlled, and characterized. Full adherence to cGMP regulations is expected to develop as the clinical trial advances. For example, process validation, methods validation, in-process testing, and establishment of specifications should be incorporated as part of the stepwise approach to fulfilling regulatory requirements.

B. Product Comparability

Changes in the manufacturing process, equipment, or facilities often occur during product development and can result in change in the biological product itself. A manufacturer must fully describe any change to a biological product in the IND or license application, regardless of whether the change occurs prior to or after product approval. The manufacturing change should be assessed and the resulting product compared with the existing product to assure that the change does not alter the safety, purity, potency, or integrity of the final product. Determinations of product comparability may be based on a combination of in vitro or in vivo studies ranging from chemical, physical, and biological assays to assessment of pharmacokinetics and/or pharmacodynamics and toxicity in animals, to clinical testing. The type of study required would depend on the extent of the change and the phase of clinical development in which the change occurs. Product comparability should be demonstrated through side-by-side analyses of the product lots manufactured under the established procedures and qualification lots of the product manufactured by the new procedure. If a sponsor can demonstrate comparability with nonclinical data, additional clinical safety, and/or efficacy trials with the new product generally will not be needed. The FDA will determine if comparability data are sufficient to demonstrate that additional clinical studies are unnecessary. Example of changes that would require a comparability study include any change in the manufacturing scheme or site, changes to the master cell bank, modification of the vector product, a change in fermentation, isolation or purification, change in storage container, or product formulation. The final section of this chapter contains references that provide additional guidance on the demonstration of product comparability.

V. GUIDANCE DOCUMENTS AND OTHER REFERENCES

CBER Documents Relevant to Gene Therapy

All current and past regulatory documents can be obtained from the CBER website: www.fda.gov/cber/publications or by emailing CBER_info@CBER.FDA.Gov.

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Guidance for Industry, Stability Testing of Drug Substances and Drug Products, Draft Guidance, FDA, CDER, CBER, June 1998.

Revisions to the General Safety Requirements for Biological Products: Companion Document to Direct Final Rule, *Federal Register* Vol. 63, No. 75, April 20, 1998.

Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors, October 18, 2000.

Draft Guidance for Industry: INDs for Phase 2 and 3 Studies of Drugs, Including Specified Therapeutic Biotechnology-derived Products, Chemistry Manufacturing and Controls Content and Format, April 20, 1999.
Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines), November 2001, 66 FR 57970.
Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices, 1987.
International Conference on Harmonization: Guidance on Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin, *Federal Register* Vol. 63, Number 185, September 24, 1998.
ICH document Q5D, Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products.
ICH Harmonized Tripartite Guidelines, ''Impurities: Guidance for residual Solvents,'' ''Impurities in New Drug Substances,'' and ''Impurities in New Drug Products.''
ICH document Q5C Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products, November 1995.
Additional information regarding gene therapy clinical trials and ORDA submission requirements can be found at the ORDA, NIH homepage: http://www.nih.gov/od/orda/.
Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines), April 1998.

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42

Immunoisolated Cells as Gene Therapy for CNS Diseases

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I. INTRODUCTION

Degenerative central nervous system (CNS) diseases are characterized by the continuous deterioration of cognitive and motor functions leading to prolonged periods of increasing incapacity. Among the most problematic and prevalent neurological disorders are those associated with the loss of specific populations of brain neurons. Approximately 12 million people in the United States suffer from such neurological disorders resulting in public expenditures and secondary medical expenses that exceed $400 billion annually (1). Beyond monetary costs to the health care economy, however, the medical, societal, familial, and personal costs cascading from these diseases defy calculation.

Advances in molecular biology, genetic engineering, proteomics, and genomics are making available an increasing number of proteins, peptides, and other compounds with enormous treatment potential. Most of these compounds are, however, not active following systemic administration, largely because the brain uses the blood–brain barrier (BBB) to modulate the local and global exchange between the vasculature and brain parenchyma. The BBB provides an exquisite regulation of the internal chemical environment of the CNS by regulating the internal environment with a mechanism of low passive permeability, combined with a highly selective transport system between the blood and the brain (2,3). It has also been a source of frustration for researchers and clinicians searching for a means of introducing drugs to the brain.

A number of strategies have been described to circumvent the BBB. Some of the techniques currently available for delivering therapeutic molecules directly into the brain include (1) carrier- or receptor-mediated transcytosis (4,5); (2) osmotic opening (6,7); (3) direct infusion with stereotactic guidance (8–10); (4) osmotic pumps (11,12); (5) sustained-release polymer systems (13–15); (6) cell replacement/cell therapy (16–18); and, (7) direct gene therapy (19–22). A presentation outlining these techniques was recently reviewed (23). In recent years, one iteration of cell-based therapy proposes to use xenogeneic cells that are encased within a selectively permeable polymeric membrane, known as immunoisolation. Immunoisolation was originally described in 1933 by V. Bisceglie (24) with the demonstration that encapsulated xenograft cells survived beyond the limit for humoral rejection. The further application of immunoisolation for the CNS owes much of its foundation to investigators focused on peripheral diseases, particularly diabetes (25,26) and Parkinsons's disease (PD), using dopamine secreting cells (27). Immunoisolation is based on the observation that xenogeneic cells can be protected from host rejection by encapsulating, or surrounding them within an immunoisolatory, semipermeable membrane. Single cells or small clusters of cells can be enclosed within a selective, semipermeable membrane barrier which admits oxygen and required nutrients and releases bioactive cell secretions, but restricts passage of larger cytotoxic agents from the host immune defense system. The selective membrane eliminates the need for chronic immunosuppression of the host and allows the implanted cells to be obtained from nonhuman sources, thus avoiding the constraints associated with cell sourcing, which have limited the clinical application of unencapsulated cell transplantation.

In this chapter, I track the use of immunoisolated cells from the initial cell biology and encapsulation process, through several preclinical research models, and ultimately to human clinical trials (Fig. 1). The preclinical animal model data demonstrating the therapeutic potential of genetically modified, encapsulated cells for Alzheimer' disease (AD), PD, and Huntingtons's disease (HD) will be highlighted. Finally, the current state of initial clinical trials using encapsulated cells therapy is discussed.

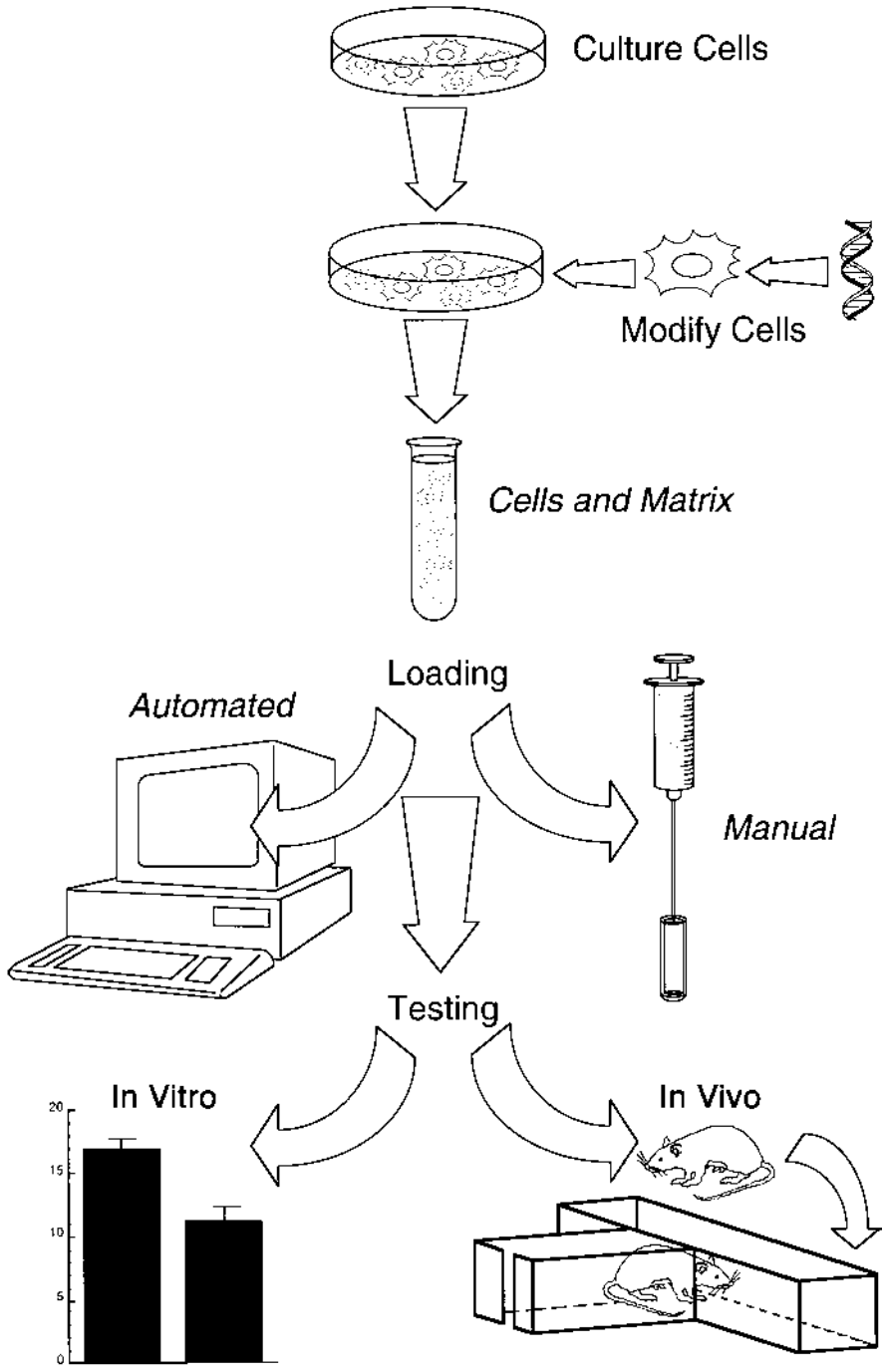

Figure 1 Flow diagram illustrating the steps involved in using genetically modified polymer encapsulated cells for CNS implantation. Cells are cultured and modified in vitro prior to either manual or automated loading into hollow fiber membranes. Following in vitro characterization, the cells are transplanted into the desired region of the brain and in vivo tests are conducted to determine the potential efficacy produced by the products secreted from the encapsulated cells.

II. CELL IMMUNOISOLATION

There are generally 2 categories for cell immunoisolation by encapsulation, micro- and macro-, each with some benefits and limitations (Table 1).

A. Microencapsulation

Microencapsulation involves surrounding cells with a thin, spherical, semipermeable polymer film. The small size, thin wall, and spherical shape of microcapsules is structurally optimal for diffusion, cell viability, and release kinetics. However, in certain types of thermoplastic-derived processing to form the microcapsule membrane around cellular clusters, cells can be exposed to organic solvents (28). Other types of microcapsules (e.g., the polyelectrolytes), although nontoxic during processing, are generally mechanically fragile and chemically unstable. For the polyelectrolytes, microcapsule membranes are formed by ionic or hydrogen bonds between 2 weak polyelectrolytes with opposite charges. Microcapsules can be prepared by gelling droplets of a polyanion/cellular suspension (e.g., alginate) in a mixing divalent cationic bath, which immobilizes the cells in a negatively charged matrix, and then coating the immobilized cells with a thin film of a polycation, such as poly(L-lysine). The poly(L-lysine)-alginate bond creates a permselective membrane whose molecular mass cutoff is on the order of 30,000 to 70,000 daltons (29). Because poly (L-lysine) is not biocompatible, a second layer of alginate is generally added to the capsule surface (Figs. 2 and 3). Polyelectrolyte-based techniques have the advantage of avoiding organic solvents and, with proper permeability control, microcapsules represent an effective configuration for cell viability and neurochemical diffusion. These types of microcapsules are, however, mechanically and chemically fragile, as well as difficult to retrieve.

B. Macroencapsulation

Macroencapsulation involves filling a hollow, usually cylindrical, selectively permeable membrane with cells, generally suspended in a matrix, and then sealing the ends to form a capsule [reviewed in (30,31)]. Polymers used for macroencapsulation are biodurable, with a thicker wall than that found in microencapsulation. Although thicker wall and larger implant diameters can enhance long-term implant stability, these features may also impair diffusion, compromise the viability of the tissue, and slow the release kinetics of desired factors. In theory, macroencapsules can be retrieved from the recipient and replaced, if necessary.

Macroencapsulation is generally achieved by filling preformed thermoplastic hollow fibers with a cell suspension. The hollow fiber is formed by pumping a solution of polymer in a water-miscible solvent through a nozzle concurrently with an aqueous solution. The polymer solution is pumped through an outer annular region of the nozzle, whereas the aqueous solution is pumped through a central bore. Upon contact with the water, the polymer precipitates and forms a cylindrical hollow fiber with a permselective inner membrane or ''skin.'' Further precipitation of the polymer occurs as the water moves through the polymer wall, forcing the organic solvent out and forming a trabecular wall structure. The hollow fiber is collected in a large aqueous water bath, where complete precipitation of the polymer and dissolution of the organic solvent occurs. The ends of the hollow fiber are then sealed to form macrocapsules. This final step is not a trivial one because reliably sealing the ends of capsules can be extremely difficult

Table 1 Advantages and Disadvantages of Unencapsulated, Micro- and Macroencapsulated Implants

Unencapsulated implants	Microencapsulation	Macroencapsulation
Advantages		
Permits anatomical integration between the host and transplanted tissue	Permits use of allo- and xenografts without immunosuppression	Permits use of allo- and xenografts without immunosuppression
Good cell viability and neurochemical diffusion	Thin wall and spherical shape are optimal for cell viability and neurochemical diffusion	Reasonable mechanical stability
		Adequate cell viability and neurochemical diffusion
		Retrievable
Disadvantages		
Likely require immunosuppression	Mechanically and chemically fragile	Dimensions (i.e., diameter and wall thickness) may limit neurochemical diffusion and cell viability
Tissue availability limited	Multiple implant sites	Multiple implant sites
Limited retrievability	Limited retrievability	May produce significant tissue damage/displacement during implantation
Societal and ethical issues		

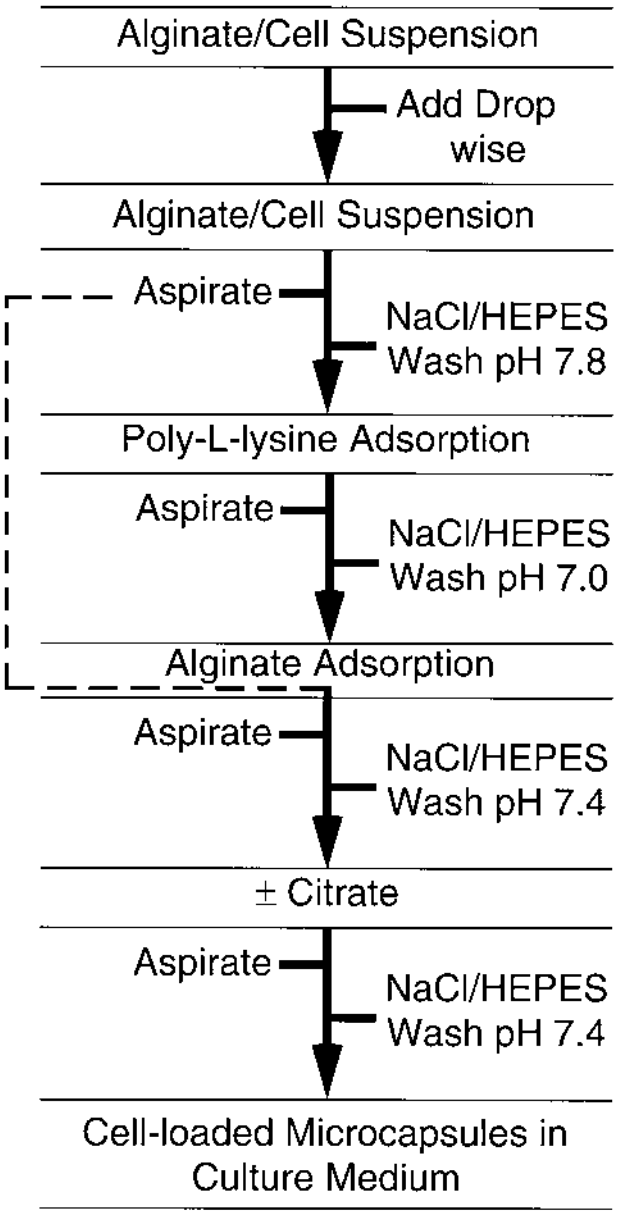

Figure 2 Flow diagram outlining the steps involved in the microencapsulation of cells. The poly(L-lysine) and alginate are sequentially adsorbed onto the alginate microspheres following immersion in a water bath.

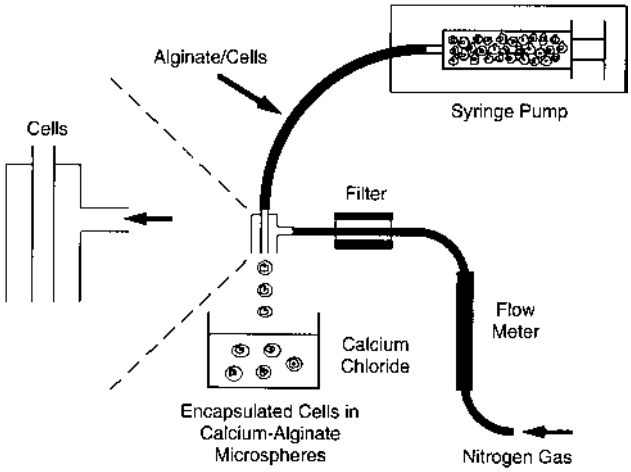

Figure 3 Schematic illustration of a syringe pump extrusion technique for encapsulating cells within alginate microcapsules. The alginate/cell suspension is extruded through the center of the annular spinneret, whereas filtered nitrogen is passed through a surrounding outer tube. In this manner, droplets are sheared off the spinneret assembly and dropped into a container of $CaCl_2$.

and provides the barrier paramount for successful immunoisolation.

A second method of macroencapsulation, called coextrusion, avoids the sealing problem by entrapping cells within the lumen of a hollow fiber during the fabrication process (32). Pinching the fiber before complete precipitation of the polymer causes fusion of the walls, providing closure of the extremities while the cells are inside. The advantages of coextrusion over loading preformed capsules are that the cells are distributed more uniformly along the entire length of the fiber and shear stresses on the cells are reduced during the loading process. In addition, the coextrusion process offers the potential for mass production of cell-loaded capsules.

Several modifications to macrocapsule configurations have been designed to provide added strength to ensure device integrity. It is important for the encapsulating membrane to exhibit enough compliance to meet the dynamics of the surrounding tissue, thus not eliciting a foreign body reaction, yet mechanically resilient to resist failure during device implantation/retrieval. Mechanical supports to provide added strength to the membrane/device are better served from within the device to therefore not impede diffusion between the encapsulating membrane and the surrounding tissue. One approach includes the addition of a cross-linked hydrogel (e.g., a 2% alginate solution) within the device. This modification was observed to enhance structural support during the implantation procedure (33). However, with time the hydrogel loses structural integrity and does not provide added strength, especially with regard to tensile strength, an important consideration for device retrieval.

The ability to retrieve devices, or remove the cellular contents from within, are important safety considerations for potential complications such as brain edema following implantation, contamination, dosing modifications, or patient request. Early designs were based on a semipermeable receptacle (16) or U-shaped configuration (32) attached to the host skull. These configurations offered the ability to empty and refill the devices, or retrieve the devices themselves, without dis-

rupting the host brain with additional neurosurgical procedures. However, devices secured to the skull could exhibit mechanical perturbations to the host tissue due to mechanical noncompliance and elicit an elevated foreign-body reaction from the host. Other configurations with internal supports have included titanium wires and braided materials to provide added tensile strength during device retrieval. Devices incorporating a titanium wire could be associated with mechanical noncompliance within the device, rendering the flexible membrane susceptible to damage. In contrast, the incorporation of braided materials within the tubular device has provided a material compliant with the membrane that enhances the tensile strength of the device.

III. CELLS AND EXTRACELLULAR MATRICES USED IN ENCAPSULATION

Cells placed within encapsulation devices generally fall into 1 of 3 categories. The first category is represented by primary postmitotic cells such as islets of Langerhans for diabetes, adrenal chromaffin cells for chronic pain, or hepatocytes for liver devices. Second, immortalized (or dividing) cells such as PC12 cells have been used to deliver dopamine for PD. The third category is typically cell lines that have been genetically engineered to secrete a bioactive substance such as baby hamster kidney (BHK) cells to secrete factors like human nerve growth factor (hNGF) for a potential therapy in AD. Dividing tissue has advantages over postmitotic tissue; it can be expanded, banked, and thus more easily tested for sterility and contaminants. However, dividing tissue is also constrained by the potential for overgrowth within the capsule environment, resulting in an accumulation of necrotic tissue that could potentially diminish the membrane's permeability characteristics, further reducing cell viability and neurochemical output.

In vivo, extracellular matrices (ECMs) provide control of cell function through the regulation of morphology, proliferation, differentiation, migration, and metastasis (34–36). Within a capsule, ECMs were originally employed simply to prevent aggregation of cells (immobilization) and resultant central necrosis, but have since been found to be beneficial to the viability and function of cells that require immobilization, as well as a scaffolding for anchorage-dependent cell lines. For example, adrenal chromaffin cells have been immobilized in alginate to prevent aggregation, which in turn reduces central necrotic cores from forming (37). The chromaffin cells appear to thrive in alginate, whereas mitotically active fibroblasts do not. In this case, the use of alginate is essential to the optimal functioning of this device because some anchorage-dependent cells such as fibroblasts or endothelial cells are present with the adrenal chromaffin cells. In the absence of alginate or similar immobilizing matrices, the fibroblasts can expand and overgrow the encapsulated milieu, resulting in a device deficient in bioactive factors produced from the chromaffin cells (38). In contrast, BHK cells, a fibroblastic cell line, prefer collagen, whereas PC12 cells exhibit a preference for distribution within precipitated chitosan, which provides a scaffolding structure on which the cells anchor (39). Inaddition, chitosan has been shown to be an effective substrate for the attachment and spreading of fibroblasts (40).

To provide a substratum designed for more specific functions, the matrix material can be manipulated chemically or mechanically, which in turn may influence cell attachment, differentiation, and/or proliferation. For example, peptides, such as arginine-glycine-aspartic acid (RGD) have been immobilized on a variety of surfaces to promote cell adhesion (41). Integrin receptors on the cell surface membrane interact with the RGD sequence, a known ligand for fibronectin receptors. Glass microbeads have been modified by attaching RGD or tyrosine-isoleucine-glycine-serine-arginine (YIGSR) motifs to provide sites for cell adhesion (41). Spherical ferromagnetic beads have been coated with specific receptor ligands to mediate cell attachment (42). With competetive binding assays and a mechanical stress testing apparatus, the endothelial cell's interaction with the ECM receptor, integrin beta-1, supported a force-dependent stiffening response, whereas nonadhesion receptors did not. A laminin-derived oligopeptide sequence, CDPG-YIGSR, has also been derivitized within an agarose hydrogel and permitted a dose-dependent increase in neurite outgrowth of neuronal cell bioassays (43). Similarly, YIGSR and isoleucine-lysine-valine-alanine-valine, both of which are found in laminin, have been immobilized on surfaces to promote neuronal cell adhesion and neurite outgrowth (44). Poly(ethylene oxide) (PEO)-star copolymers have been fabricated as a potential synthetic extracellular matrix (45). The star copolymers provide many hydroxyl groups where various synthetic oligopeptides can be attached to desired specifications.

The survival and differentiation of encapsulated cells can be influenced by the matrix interactions. A variety of matrices for use in immunoisolatory devices, such as alginate, rat tail collagen extracts, gelatin shards, porous gelatin or collagen microcarriers, carrageenan, chondroitin sulfate, fibrin, hyaluronic acid, the positively charged substrate chitosan, and an acrylamide-based thermoresponsive gel are available and were recently reviewed (46). All in all, successful cell encapsulation involves the choice of the cells to be encapsulated, the type of intracapsular matrix used, and the ability to control membrane properties such as geometry, morphology, and transport (Fig. 4). The interactions between the encapsulating membrane characteristics and the capsule core, or matrix within, should be rigorously characterized to determine the optimal configuration for each cell type.

IV. PREPARATION OF IMMUNOISOLATORY MEMBRANES

The majority of thermoplastic ultrafiltration (UF) and microfiltration (MF) membranes used to encapsulate cells are manufactured from homogenous polymer solutions by phase inversion (47). Ultrafiltration membranes have pore sizes ranging from 5 nm to 0.1 μm, while microfiltration (or microporous) membranes have pores ranging from 0.1 to 3 μm. Phase inver-

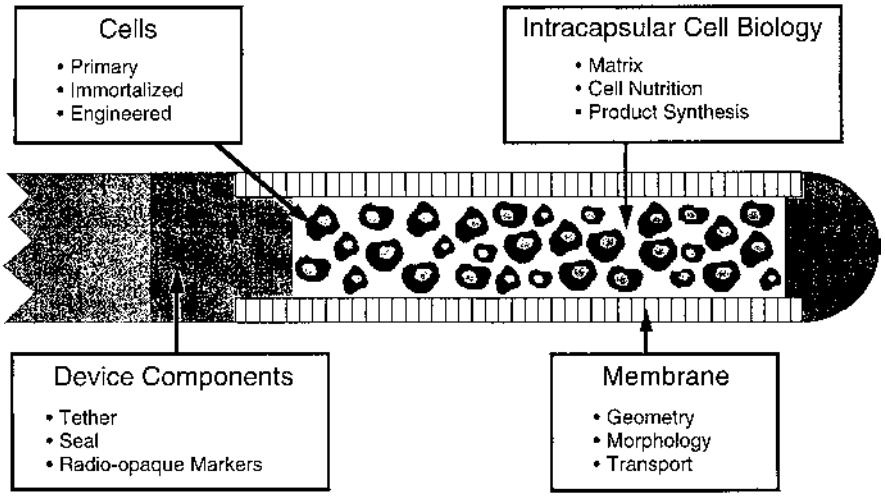

Figure 4 Diagram illustrating the different components of a macrocapsule contributing to successful implantation and cell viability. The manufacturing process involves several different aspects each with its own complexities. The initial choice of cell types includes primary, immortalized, or engineered. Intracapsular cell biology issues following encapsulation include a consideration of the need to use a compatible extracellular matrix and other considerations specific to that cell type because they impact cell nutrition and product synthesis. A series of other device-related issues include membrane geometry, morphology, and transport of molecules into and out of the device. Finally, the device must be sealed and, depending on the site of implantation, could require a tether for subsequent retrieval or the inclusion of radio-opaque markers for imaging purposes.

sion is a versatile technique that allows for the formation of membranes with a wide variety of nominal molecular weight cutoffs, permeabilities, and morphologies. The morphology and membrane properties depend on the thermodynamic parameters and kinetics of the fabrication process. In phase inversion, the polymer is first dissolved in an appropriate solvent. The solution is cast as a flat sheet or extruded as a hollow fiber. As part of the casting or extrusion procedure, the polymer solution is precipitated by a phase transition, which can be brought about by changing the temperature or solution composition. This process involves the transfer of a single-phase liquid polymer solution into a 2-phase system consisting of a polymer-rich phase that forms the membrane structure, and a second liquid polymer-poor phase that forms the membrane pores. Any polymer that will form a homogenous solution, which under certain temperatures and compositions will separate into 2 phases, can be used. Thermodynamic and kinetic parameters, such as the chemical potential of the components and the free energy of mixing of the components, determine the manner in which the phase separation takes place (48).

In cases where membrane strength limits the overall device strength, the membrane must be manufactured with certain considerations in mind, and the membrane dimensions, composition, and structure may have to be altered. Choosing a material that is inherently stronger (i.e., more ordered), or higher in molecular weight, with which to cast the membrane should increase the overall mechanical properties. UF or MF membranes can be fabricated with macrovoids within the wall or as an open-cell foam where the microvoids are interconnected. By incorporating techniques that increase the isoreticulated structure within the membrane wall, the tensile strength can be increased with similar membrane porosity, thus maintaining the same overall diffusive transport. The strength also can be improved by increasing the cross-sectional area of the membrane by thickening the walls. Decreasing the overall membrane porosity increases the overall membrane strength. An example of a macrovoid-containing structure is presented in Fig. 5.

The outer morphology of the membranes can be altered during fabrication or by a posttreatment to improve the host tissue reaction. Using various phase inversion techniques, the outer surface of the membrane can range from a selectively permeable membrane ''skin'' to a structure large enough to allow cells to enter the wall (approximately 10–20 μm in diameter). The combination of proper membrane transport and outer morphologies may also be achieved with composite membranes (49).

V. MEMBRANE TRANSPORT CHARACTERIZATION

Membrane transport is typically characterized by the capability to retain marker molecules in convective sieving experiments. Membrane transport properties have been extensively reviewed elsewhere (31,46,50).

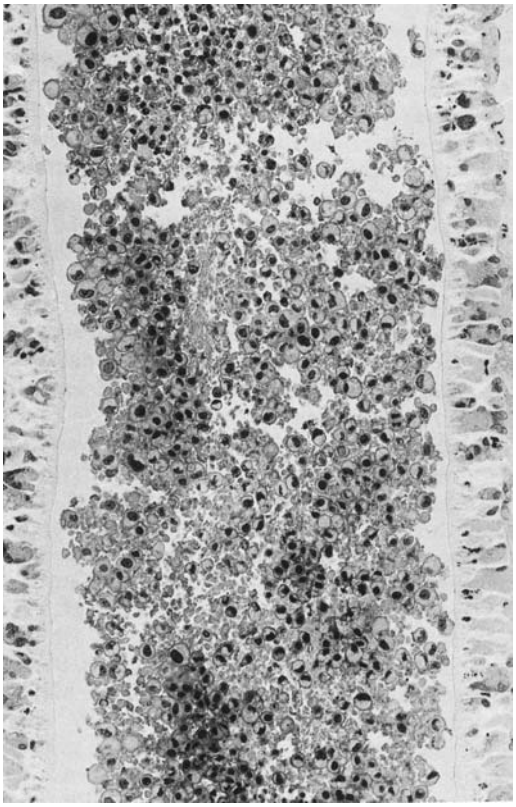

Figure 5 An example of encapsulated fibroblasts retrieved from monkey lateral ventricle illustrating many of the components listed above. Cells were modified to produce NGF and were encapsulated using Vitrogen as an extracellular matrix. Under these conditions, numerous H&E-positive cells were visible and evenly distributed within the full length of the capsule 1 month following implantation.

VI. VALIDATION OF THE CONCEPT OF IMMUNOISOLATION

A. In Vitro Studies

Two general approaches have been used to validate the premise that encapsulated cells are indeed immunoisolated. The maintenance of immunoisolation (i.e., capsule integrity) has been evaluated by several methods. Initial integrity was characterized by encapsulating a polydisperse (10^3–10^6 g/mol) dextran solution and monitoring the flux of the molecules across the semipermeable membrane over time. Similar to what has been described for assessing the molecular weight cutoff of hollow fiber membranes, a dextran rejection curve was measured from the filtrate and reservoir concentrations measured by gel permeation chromatography (50). With controlled devices that had been damaged, the large molecular weight dextran species were observed within the reservoir concentrations, while intact, integral capsules did not demonstrate these observations. In addition, capsule integrity has been evaluated in vitro on PC12 cell-loaded macrocapsules (30). This system tested the capability of the selectively permeable membrane to protect encapsulated PC12 cells against the cytotoxic killing of antibody (IgG)-mediated complement lysis. With integral cell-loaded capsules, in the presence of antibody and complement, the capsular membrane prevented antibody-mediated complement lysis (<10% cell death), while complete killing (100%) was observed in cases of damaged capsules or with PC12 cells not encapsulated.

B. In Vivo Studies

The importance of polymer capsule integrity for xenografted PC12 cell survival is illustrated in studies where unencapsulated PC12 cells, or cells encapsulated in breached (intentionally damaged) membranes, have been implanted in the brains of guinea pigs (i.e., a xenograft) (32). Intact PC12 cell-loaded capsules implanted into the guinea pig striatum showed no lymphocytic infiltration and a minimal astrocytic reaction by GFAP staining. In contrast, cell survival was poor in capsules that were intentionally damaged, with marked inflammation and heavy lymphocytic invasion into the capsule. Moreover, unencapsulated PC12 cells do not survive following implantation into either the guinea pig or the nonhuman primate striatum, whereas encapsulated PC12 cells have demonstrated viability for 6 months in nonhuman primates (51).

In addition, long-term studies were undertaken to evaluate the ability of a rat to generate antibodies against bovine adrenal chromaffin cells when implanted in the ventricular space (52). There was no evidence of elevated levels of rat antibovine adrenal chromaffin cell IgG or IgM levels in serum from rats implanted with encapsulated xenogeneic adrenal chromaffin cells for nearly 1.5 years in vivo. In contrast, a robust host immune response was induced in all animals at 5 months after implantation with unencapsulated bovine adrenal chromaffin cells.

Although these studies provide some level of comfort for the immunoisolatory nature of permselective membranes, very few studies have actually using encapsulated cells actually determined whether the procedure used formed a truly immunoisolatory membrane. Conceivably, the membranes used may have had molecular weight cutoffs sufficiently high enough to alter the function and viability of the cells.

VII. BIOCOMPATIBILITY

Transplant survival, with and without an encapsulating membrane, is mediated by many factors. The cellular/tissue reaction generated by a host in response to a foreign body, typically referred to as biocompatibility, impacts the success of the transplant. Factors that affect biocompatibility can include the method of implantation (53), the implant site (54), and implant properties, such as composition of the polymer, potential residual processing agents, surface integrity and microgeometry, and the size and shape of the implant (54). The constit-

uents of the implants should be assessed rigorously, both in vitro and in vivo, to determine the safety of the materials. For implants into the brain, the CNS tissue is not only privileged from an immunologic standpoint, but also lacks the primary reactive cells, fibroblasts, and macrophages found in peripheral locations. The brain, therefore, offers a unique environment both in terms of the inflammatory response, as well as the cellular constituents that comprise the reactive cells. Immunospecific antibodies are available to delineate the roles of the brain reactive cells, the astroctyes, and microglia, with respect to their reactivity. Nevertheless, few studies have systematically examined the reaction of host brain tissue to the presence of polymeric devices.

Early investigations used electron microscopic techniques to characterize the brain tissue reaction to plastic-embedded metal electrodes and polymer implants (55–57) reported in detail on the biocompatibility of a thermoplastic-based macrocapsule implanted into brain tissue. Necrosis of the tissue surrounding the polymer capsules implanted into the striatum of rodents was minimal, and small Nissl-positive cells were observed invading the open trabeculae in the wall of the macrocapsules. Immunospecific analysis for glial fibrillary acid protein indicated that reactive astrocytes were present 1 to 2 weeks after implantation, with the intensity of this reaction diminishing with time. By 4 weeks after implantation, there was a minimal gliotic reaction surrounding the polymer implant. No significant changes in myelin basic protein reactive oligodendroglia were observed, and neuron-specific enolase reactive neurons were readily identifiable adjacent to the implant. Subsequent studies with an immunospecific antisera against rat microglia, OX-42, has revealed a reaction and time course similar to the astrocytes. Last, electron microscopic analysis further revealed cellular and capillary invasion into the trabecular networks of the polymer walls.

The capillary invasion provides an ample supply of nutrients and oxygen in proximity to the encapsulated cells. However, because the process of angiogenesis for neovascularization typically evolves in a 4 to 7 day period (58), the encapsulated cells must endure an initial period of nutrient and oxygen deprivation. In addition, because the only means of delivery of the desired cellular products from an encapsulated cell implant is by diffusion, any reaction around the capsule might diminish the diffusion of therapeutic products from the encapsulated cells and could compromise the viability of the encapsulated tissue. These studies clearly demonstrated biocompatibility within the host nervous system, which suggests that the bidirectional transport of low molecular weight solutes across the permselective membrane can be maintained in vivo. However, these studies were undertaken with empty polymer macrocapsules (57). Cells enclosed within the capsule can impact device biocompatibility. The inclusion of primary tissues, such as bovine adrenal chromaffin cells, typically results in a host tissue interface equivalent to that observed for the empty capsules. In contrast, PC12 cell-loaded capsules can elicit a more robust host response, but additionally, have increased the level of angiogenesis adjacent to the implant. Enhancing angiogenesis has also been investigated by releasing VEGF from planar diffusion chambers to induce vascularization of the transplanted devices (59).

In an effort to further enhance the biocompatibility for cell line-containing implants, or reduce protein adsorption that may negatively impact the ability to maintain adequate long-term diffusive characteristics, several postsynthesis modifications have been attempted. Poly(acrylonitrile-co-vinyl chloride) (PAN/VC) hollow fiber membranes, which were surface modified by grafting poly(ethylene oxide) (PEO) groups, exhibited improved biocompatibility in brain tissue over the unmodified PAN/VC controls (60). Similar observations were made with PEO-modified poly(hydroxyethyl methacrylate-co-methyl methacrylate) membranes used extensively in cellular microencapsulation (28).

VIII. LONG-TERM PRODUCT SECRETION AND DELIVERY

Before patients can be routinely implanted with encapsulated cells, long-term survival (>6 months) of the encapsulated cells and continued release of the therapeutic molecule must be demonstrated. Although effective immunoisolation should result in long-term survival of encapsulated cells, surprisingly few studies have examined long-term (>3 months) implant viability. A few notable exceptions exist and provide compelling evidence about the potential for long-term survival and release of molecules from the cells. Encapsulated PC12 cells secrete high levels of catecholamines under both basal and evoked conditions, and have been evaluated as a potential means of delivering dopamine to the striatum of PD patients. Following encapsulation, PC12 cells have been maintained in vitro and in vivo for at least 6 months, while maintaining a typical morphology and clustered arrangement along the lumen of the device (32,51,61,62). The cells remain tyrosine hydroxylase (TH)-immunoreactive and can be seen in various stages of mitosis, with some necrosis in regions of high cell density. The continued presence of TH suggests that the encapsulated cells maintain the ability to synthesize and release dopamine, and indeed spontaneous and evoked release of dopamine can be detected from capsules maintained both in vitro, and following explantation from the CNS. Electron microscopy confirms the presence of numerous mitochondria, polysomes, and electron-dense secretory vesicles distributed within the cytoplasm. Both rodent microdialysis and positron emission tomography studies in primates have confirmed that encapsulated PC12 cells continue to produce L-DOPA/dopamine in situ (62,63). Other cell types, specifically bovine adrenal chromaffin cells, have been reported to survive for prolonged periods of time following encapsulation. The long-term survival of bovine adrenal chromaffin cells in macrocapsules has been characterized in spinal implants to provide a sustained source of pain-reducing neuroactive substances (64). The most convincing demonstration of long-term survival of chromaffin cells comes from a study in which intraventricular implants of encapsulated bovine chromaffin cell implants survived for nearly 1.5 years and continued to pro-

duce catecholamines and met-enkephalin (52). These results indicated the capacity for long-term survival of encapsulated xenogeneic cells implanted in the lateral ventricles of rats. Similar observations in a higher species have not been obtained.

One of the more interesting areas of research involves using both encapsulated and unencapsulated cells as biological systems for delivery of growth factors. Encapsulated cells have been used to deliver nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), neurotrophin 4/5 (NT 4/5) and glial-derived neurotrophic factor (GDNF), but only the long-term effects of NGF have been examined. Polymer-encapsulated cells have been reported to survive and continue to secrete NGF for 13.5 months in normal rodents (65). Following the 13.5-month implant interval, the NGF transgene copy number, as measured with polymerase chain reaction (PCR) analyses, from the recovered NGF cells was equivalent to preimplant levels, indicating NGF gene stability. In addition, as measured by enzyme-linked immunosorbent assay (ELISA), the NGF released from encapsulated cells into the tissue culture medium was 64% higher following removal from the rat lateral ventricles after the 13.5-month interval in situ. No deleterious effects from NGF were detectable on body weight, mortality rate, motor/ambulatory function, or cognitive function as assessed with the Morris water maze and delayed matching to position in healthy young adult rats. In addition, there was no evidence that NGF from these encapsulated cells produced hyperalgesia, although tests of somatosensory thresholds did reveal effects related to the NGF delivery. Morphologic analysis of retrieved cell-loaded devices revealed abundant viable BHK-NGF cells throughout the capsule. These same animals exhibited a marked hypertrophy of cholinergic neurons within the striatum and nucleus basalis, as well as a robust sprouting of cholinergic fibers within the frontal cortex and lateral septum proximal to the implant site. Although no deleterious behavioral effects were observed, the profound anatomical changes and their relationship to functional alterations in normal and diseased brain warrant additional study.

IX. HOST-SPECIFIC EFFECTS ON OUTPUT OF ENCAPSULATED CELLS

The available data also suggest substantial variability in the in vivo performance of encapsulated cells even during the first few months. For example, there was a large range in dopamine and L-DOPA output from rodent-size devices, from 0 to more than 50 pmol/device (66). In fact, 15% of the devices from that study had no detectable output after only 4 months in vivo. Similar variability in device performance has been observed in the majority of in vivo studies. In a primate study that produced therapeutic effects in two-thirds of the MPTP monkeys implanted with PC12 cells, all 5 devices implanted in 1 monkey had virtually 0 output after explantation, whereas all 5 devices implanted in another monkey had relatively high output and all 5 devices in the third monkey had catecholamine output in the midrange (51). These results clearly demonstrate that some of the variability in device performance may be atributable to individual differences between hosts, a result consistent with that reported for NGF output from encapsulated BHK cells implanted into the lateral ventricle of rodents (67). The exact mechanism for these individual differences remains undetermined but deserves serious attention.

X. DIFFUSION OF MOLECULES FROM POLYMER DEVICES

Little work has been done to date to determine the extent of diffusion of molecules from encapsulated devices into the host tissue. Winn et al. (16) initially evaluated dopamine diffusion in a denervated striatum with acute microdialysis from a dopamine releasing polymer rod placed in a semipermeable receptacle. Morphologically, the microdialysis probes were found to be located approximately 0.5 mm from the receptacle. In addition, Tresco et al. (62) conducted a series of in vivo experiments to elucidate the relationship between diffusion of dopamine from encapsulated PC12 cells and behavioral recovery in dopamine-depleted rodents. Dopamine was detectable 200 μm from PC12 cell-loaded macrocapsules, as determined by microdialysis, in concentrations similar to those obtained in unlesioned control striatum. In contrast, the levels of dopamine in the perfusate of animals that did not exhibit behavioral recovery were undetectable.

Immunocytochemistry was used to estimate the diffusion of NGF from encapsulated cell implanted into the striatum of rats (68). One month after implantation, the diffusion of NGF was estimated to be approximately 1 mm. CNTF has also been reported to be detectable in the cerebrospinal fluid (CSF) of amyctroplate scher (ALS) patients who have received intrathecal implants of encapsulated CNTF-producing BHK cells. In contrast, CSF levels of NGF were not detectable in nonhuman primates that received intraventricular grafts of NGF-producing cells (69). At best, these data suggest that diffusion of molecules from encapsulated cells is limited. Moreover, the majority of degenerative CNS diseases will likely not be treatable by delivering drugs from the ventricular space given that diffusion of compounds in CNS tissue is generally severely limited when only governed by passive diffusion. Future work, particularly clinical considerations, must seriously consider this issue when determining the optimal numbers and spacing of polymer devices.

XI. ENCAPSULATED CELLS FOR PARKINSON'S DISEASE

A. Effects of Catecholamine-producing Cells

PD is an age-related neurodegenerative disorder characterized by hypokinesia, rigidity, and tremor. These behavioral abnormalities are mediated by a marked loss of dopaminergic neurons in the pars compacta of the substantia nigra. Pharmacological treatments are based on replacing or increasing striatal dopamine levels [e.g., L-dihydroxyphenylaline (L-DOPA) ad-

ministration]. Oral administration of L-DOPA significantly improves motor deficits in the early stages of PD. Unfortunately, this pharmacological approach has limitations. Systemic administration results in drug distribution to extrastriatal dopamine receptors, which may produce psychoses and vomiting. The therapeutic window of L-DOPA's beneficial effects becomes progressively limited as the disease continues its degenerative course. Pulsatile delivery seems to be associated with more adverse effects than continuous delivery (70). Studies using pumps to deliver dopamine continuously and directly to the striatum in both rodent and primate models of PD have reported improved motor function with few adverse effects (71–73). Several studies have detailed the effects of implanting encapsulated dopamine-producing cells into the striatum.

1. Rodent Studies

Several detailed studies have been conducted in both rodent and primate models of PD, which validate the potential of this approach. Rats with PC12 cell-loaded capsules implanted in a dopamine-depleted striatum exhibit fewer rotations after apomorphine administration than nonimplanted rats, which suggests that the devices are releasing catecholamines at levels sufficient to reduce the degree of synaptic supersensitivity that develops after dopamine-depleting lesions (Table 2). Within 2 weeks following implantation, the number of apomorphine-induced rotations is decreased by approximately 40% to 50% and remains at this level for up to 6 months (62). The specificity of the effects of PC12 cells is further supported by the observation that reductions in rotational behavioral do not occur following implants of empty polymer devices and that reductions in rotations are only evident in rats implanted in the denervated striatum and not in rats with devices implanted into the lateral ventricles (66,74). Furthermore, the behavioral effects persist only as long as the devices remain in the striatum.

Measures of drug-induced rotations provide a convenient method for assessing potential efficacy, and significant information has been acquired using this initial preclinical screen. However, relying exclusively on changes in drug-induced rotations has been criticized for the lack of clinical relevance and specificity. Accordingly, the effects of PC12 cells on a battery of nondrug-induced behaviors have been examined. Neurological testing revealed behavioral deficits in the affected forelimb analogous to the deficits exhibited in PD patients, and these deficits were significantly attenuated by oral Sinemet® (66,75). Because any transplantation procedure will be used as an adjunct to L-DOPA administration, these data provided the opportunity to investigate the effects of PC12 cells on both relevant behavioral measures and on the therapeutic window of oral Sinemet. Rats with severe unilateral dopamine depletions received striatal implants of encapsulated PC12 cells. Rats were evaluated on a series of behavioral tests over a range of doses of oral Sinemet. Results confirmed that cell-based delivery of L-DOPA and dopamine, directly to the denervated striatum, attenuated parkinsonian symptoms. The magnitude of the therapeutic effect produced by continuous, site-specific delivery of catecholamines was greater than the effect prodcued by acute, systemic oral Sinemet. The beneficial effects of oral Sinemet and striatal implants of PC12 cells were additive, but there were no adverse effects related to the implantation of the PC12 cells and these devices did not increase the adverse effects related to oral Sinemet (76). Therefore, striatal implants of catecholamine-producing devices have direct therapeutic effects, and perhaps more important, they appear to widen the therapeutic window of oral Sinemet.

Table 2 Behavioral Outcome from Encapsulated Catecholamine-secreting PC12 Cells in Models of PD

Animal model	Testing paradigm	Outcome[a]
Unilateral lesioned rats	Receptor-activated rotations	+
	T-maze alternation	+
	Somatosensory function	+
	Forelimb freezing/bracing	−
	Forelimb placing	−
	Forelimb akinesia	−
	Postural/locomotor forelimb use	−
Bilateral lesioned rats	Feeding/drinking	−
Aged rats	Activity levels	−
	Balance and coordination	+
MPTP-lesioned primates	Skilled hand use	+
	Clinical rating scale	+

[a] +, positive outcome; −, negative outcome.

In addition to motor deficits produced experimentally by depleting nigrostriatal dopamine systems in young rodents, motor deficits are also observed with increased age. Age-related deficits in motor functions include deficits in balance, coordinated movement, and generalized locomotion. Striatal dopaminergic systems appear to be involved integrally in the motor perturbations that occur during aging, and enhancement of striatal dopamine function in aged animals by induction of dopamine receptor up-regulation or administration of dopaminergic agonists can reduce age-related motor deficits. Because the motor deficits observed in aged rodents appear to be mediated partially by alterations in striatal dopamine systems, studies were conducted to evaluate the potential efficacy of striatal implants of polymer-encapsulated PC12 cells on age-related motor dysfunction in rats (77). In these studies, aged rats were significantly hypoactive relative to young animals. Moreover, compared with young rats, the aged rats (1) remain suspended from a horizontal wire for less time, (2) are unable to descend a wooden pole covered with wire mesh in a coordinated manner, (3) fall more rapidly from a rotating rod, and (4) are unable to maintain their balance on a series of wooden beams with either a square or rounded top of varying widths.

Following baseline testing, aged rats received bilateral striatal implants of empty capsules or PC12 cell-loaded capsules. Three weeks later, the aged rats that received PC12 cells showed a robust improvement in performance on the rotarod task and balance on the wooden beams. No recovery was observed in the animals receiving PC12 cell-loaded capsules on any of the other motor tasks (Table 2). Likewise, no improvement was observed on any behavioral measure in those animals receiving empty capsules.

2. Primate Studies

The studies conducted to date in the 6-OHDA rat model of PD generally support the clinical utility of encapsulated catecholamine-producing cells to restore dopamine levels within the denervated striatum. However, nonhuman primates are a more relevant model due to their size and complexity of the nervous system, which more closely approximates humans. This is especially important in neural transplantation because of the need for therapeutic agents to diffuse from the implant site through the striatal parenchyma. In terms of tissue volume, diffusion through a rat brain is easier to accomplish than adequate diffusion through the much larger human brain. The nonhuman primate model allows the assessment of therapeutic potential at this level in a way that cannot be approximated in rodents. Accordingly, the potential efficacy of encapsulated PC12 cells has been evaluated in a unilateral MPTP-lesioned primate model. Cynomolgus monkeys trained to perform a task that involved extending the arm and required fine use of the digits to pick food from small food wells were unilaterally lesioned with an injection of MPTP in the right carotid artery. The resulting MPTP-induced lesion produced a significant and stable impairment in the ability of the animals to use the contralateral limb to retrieve food rewards from the wells. The times required to empty the wells were measured for 3 months postlesion, and the monkeys were then implanted with a U-shaped device that was immediately filled with a suspension of PC12 cells (78). Following implantation of the cells, manual dexterity was improved and the time required for the monkeys to empty the tray using the impaired hand gradually decreased (Table 2). Although the PC12 cells attenuated the parkinsonian deficit in this task, some tremor remained and the animals did not recover to prelesion levels. Prior to the implantation of cells, the monkeys left arm was essentially immobile. After implantation, the monkeys could consistently move both their arm and use their fingers. When the cells were flushed out of the device, performance declined again and the time required to empty the tray increased toward preimplant levels. Together, these data indicated that encapsulated cells survived, were functional, and promoted behavioral recovery even in primate models of PD.

Similar results were obtained in a study by Kordower et al. (51), where 4 cynomolgus primates were trained on a skilled reaching task similar to that described above and then rendered hemiparkinsonian with an intracarotid injection of MPTP. Three animals received implants of encapsulated PC12 cells into both the caudate and putamen, and 1 animal received implants of empty capsules and served as a control. After a transient improvement, limb use in the control monkey dissipated and returned to post-MPTP levels of disability. Two of the 3 PC12 cell-implanted monkeys recovered performance on the task to near normal levels for up to 6.5 months post-transplantation. Capsules retrieved from the monkeys who recovered limb function contained abundant viable PC12 cells that continued to release L-DOPA and dopamine. In contrast, capsules retrieved from the monkey that did not recover contained few viable PC12 cells. Neuroanatomical and neurochemical evaluation of the implanted striatum failed to reveal any host-derived sprouting of catecholaminergic or indolaminergic fibers, which further suggested that the observed behavioral recovery was due to secretion of catecholamines from the encapsulated PC12 cells.

B. Effects of Neurotrophic Factor-producing Cells

Several recent studies have investigated the ability of encapsulated neurotrophic factor-secreting cells to exert neutotrophic effects in rodent models of PD (79–82). In an initial study (81), the ability of encapsulated GDNF-producing cells to prevent the degeneration of dopaminergic neurons was investigated in rats following transection of the medial forebrain bundle. Encapsulated cells releasing aproximately 5 ng of GDNF/day were implanted immediately rostral to the substantia nigra. One week later, the medial forebrain bundle was transected and the animals were tested for amphetamine-induced rotations for 1 week later. GDNF treatment significantly reduced the numbers of rotations in lesioned animals. GDNF treatment also attenuated the loss of neurons in the substantia nigra but had no effect on dopamine within the denervated striatum. Using the same model system, neuturin-producing cells, a homologue of GDNF, was investigated for its neurotrophic activity (82). Neurturin-treated animals had significantly more TH-positive neurons in the substantia nigra (51% compared with 16% in controls), but failed to show any behavioral improvement as measured by rotational behavior. Together, these data suggest that encapsulated cells may have a role in neurotrophic therapy for PD. However, additional studies in animal models are required to detemrine the relationship between the anatomical and behavioral consequences of cell-based delivery.

XII. ENCAPSULATED NGF-PRODUCING CELLS IN ANIMAL MODELS OF AD

AD affects approximately 5% of the population over the age of 65 and is the most prevalent form of adult onset dementia. With the increase in our aging population, the incidence of AD is expected to triple over the next 75 years. The most prominent feature of AD is a progressive deterioration of cognitive and mnemonic ability, which is at least partially related to the degeneration of basal forebrain cholinergic neurons. At present, treatments are ineffective for either slowing or preventing cholinergic neuron loss or the associated memory

deficits. Several converging lines of evidence indicate that NGF has potent target-derived trophic and tropic effects on cholinergic basal forebrain neurons (83,84).

A. Rodent Studies

Although no model faithfully recaptitulates the complex etiology and time-dependent loss of cholinergic neurons seen in AD patients, model systems have been developed to answer the specific question: Can NGF prevent the death of damaged cholinergic neurons following acute trauma? Initial studies determined whether encapsulated BHK cells modified to produce human NGF could prevent cholinergic neuron loss following aspiration of the fimbria/fornix (85) (Fig. 6). Rats received lesions of the fimbria/fornix followed by intraventricular implants of either NGF-producing or control (nontransfected) cells. Control-implanted animals had an extensive loss of ChAT-positive cholinergic neurons ipsilateral to the lesion that was prevented by NGF cell implants. Quantitation of ChAT-positive neurons for the 2 groups revealed that with the control capsules, 14% of the neurons remained viable on the lesioned side compared with the nonlesioned side, whereas with the NGF capsules, 88% of the cholinergic neurons were rescued.

One of the cardinal behavioral symptoms of AD is a progressive loss of cognitive ability. Just as no animal model faithfully mimics the complex etiology and pathophysiology of AD, comparable behavioral abnormalities are difficult to reproduce in animal models. However, the aged rodent shows a progressive degeneration of basal forebrain cholinergic neurons, together with marked cognitive impairments, which are partly reversible by administering NGF. Lindner et al. (67) trained 3-, 18-, and 24-month-old rats on a spatial learning task in a Morris water maze (Fig. 7). Cognitive function, as measured in this task, declined with age. Evidence of age-related atrophy of cholinergic neurons was observed in the striatum, medial septum, nucleus basalis, and vertical limb of the diagonal band. These anatomical changes were most severe in animals with the greatest cognitive impairments, suggesting a link between the 2 pathological processes. Following training, animals received bilateral intraventricular implants of encapsulated NGF or control cells. The 18- and 24-month old animals receiving NGF cells showed a significant improvement in cognitive function (Fig. 7). No improvements or deleterious effects were observed in the young nonimpaired animals. Anatomically, the NGF released from the encapsulated cells increased the size of the atrophied basal forebrain and striatal cholinergic neurons to the size of the neurons in the young healthy rats. Furthermore, there was no evidence that the NGF cells produced changes in mortality, body weights, somatosensory thresholds, potential hyperalgesia, or activity levels, suggesting that the levels of NGF produced were neither toxic nor harmful to the aged rats.

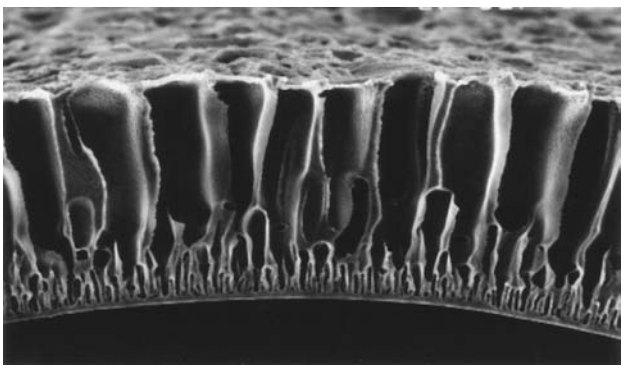

Figure 6 Scanning electron micrograph of a typical macrovoid-containing membrane used in cell encapsulation.

B. Primate Studies

Similar results were obtained in nonhuman primates (69), an essential prerequisite to human clinical trials. In these studies, cynomolgus primates received transections of the fornix followed by placement of encapsulated NGF or control cells into the lateral ventricle (Fig. 4). In the control animals, a significant reduction in the number of cholinergic neurons was observed in the medial septum and vertical limb of the diagonal band of Broca. Again, loss of cholinergic neurons was prevented by implants of NGF-secreting cells. It also appeared that cholinergic neurons within the medial septum of NGF-treated animals were larger, more intensely labeled, and elaborated more extensive proximal dendrites than those displayed by BHK-control animals (Fig. 8).

In addition to the effects on cell viability, NGF implants induced a robust sprouting of cholinergic fibers proximal to the implant site (69). All monkeys receiving NGF implants displayed dense collections of NGF receptor-immunoreative fibers throughout the entire dorsoventral extent of the lateral septum (Fig. 9). This effect was unilateral as the contralateral side displayed only a few cholinergic fibers in a manner similar to that seen in control-implanted monkeys. The cholinergic nature of this sprouting was confirmed by an identical pattern of fibers, which were both ChAT-immunoreactive and AChE-positive. These fibers ramified against the ependymal lining of the lateral ventricle adjacent to the transplant site and were particularly prominent within the dorsolateral quadrant of the septum corresponding to the normal course of the fornix. The cell sparing and sprouting results have been replicated in a group of aged nonhuman primates (86).

XIII. POLYMER-ENCAPSULATED CELLS TO DELIVER NEUROTROPHIC FACTORS IN ANIMAL MODELS OF HD

HD is an inherited, progressive neurological disorder characterized by a severe degeneration of basal ganglia neurons, particularly the intrinsic neurons of the striatum. Accompanying the pathological changes is a progressive dementia coupled

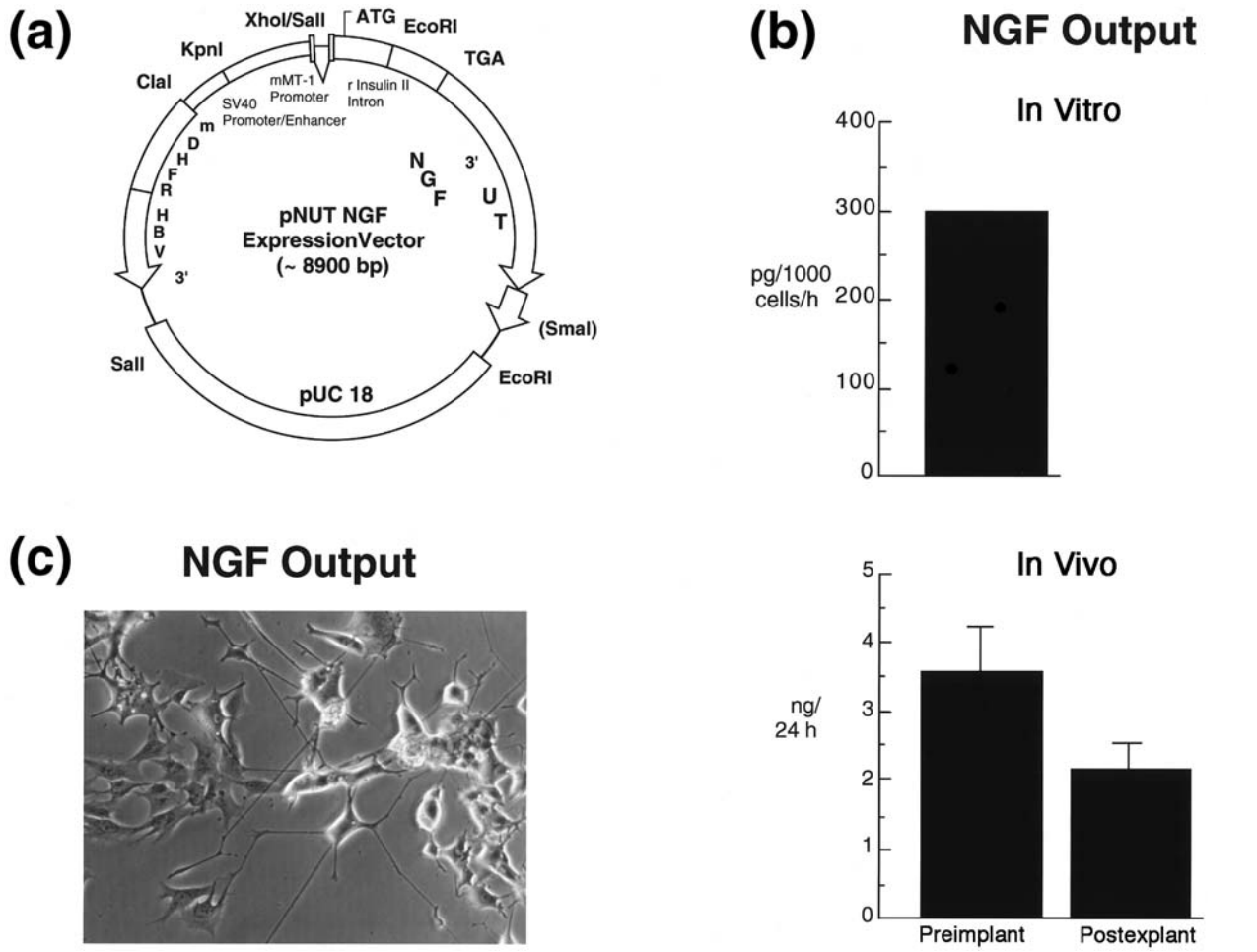

Figure 7 (a) Expression vector containing the NGF gene. (b) NGF levels, as determined by ELISA, in unencapsulated (top) and encapsulated (bottom) BHK cells. The in vivo levels were determined from devices 3 months following explant from rodent striatum. (c) The biological activity of the NGF from encapsulated BHK cells is shown in phase-contrast photomicrographs of PC12 cells that exhibit exytensive neurite processes. Original magnification, 25 um.

with uncontrollable movements and abnormal postures. From the time of onset, an intractable course of mental deterioration and progressive motor abnormalities begins with death usually occurring within 15 to 17 years. Overall, the prevalence rate of HD in the United State is approximately 50 per 1,000,000 (87). At present, there is no treatment that effectively addresses the behavioral symptoms or slows the inexorable neural degeneration in HD.

Intrastriatal injections of excitotoxins such as quinolinic acid (QA) have become a useful model of HD and can serve to evaluate novel therapeutic strategies aimed at preventing, attenuating, or reversing neuroanatomical and behavioral changes associated with HD (88–91). The use of trophic factors in a neural protection strategy may be particularly relevant for the treatment of HD. Unlike other neurodegenerative diseases, genetic screening can identify virtually all individuals at risk who will ultimately suffer from HD. This provides a unique opportunity to design treatment strategies to intervene prior to the onset of striatal degeneration. Thus, instead of replacing neuronal systems, which have already undergone extensive neuronal death, trophic factor strategies could be designed to support host systems destined to die at later time in the patient's life.

A. Rodent Studies

Infusions of trophic factors such as NGF or implants of cells genetically modified to secrete NGF have proven effective in preventing the neuropathological sequelae resulting from intrastriatal injections of excitotoxins, including QA (92–95). Emerich and colleagues (96,97) examined the ability of encapsulated trophic factor-secreting cells to affect central striatal

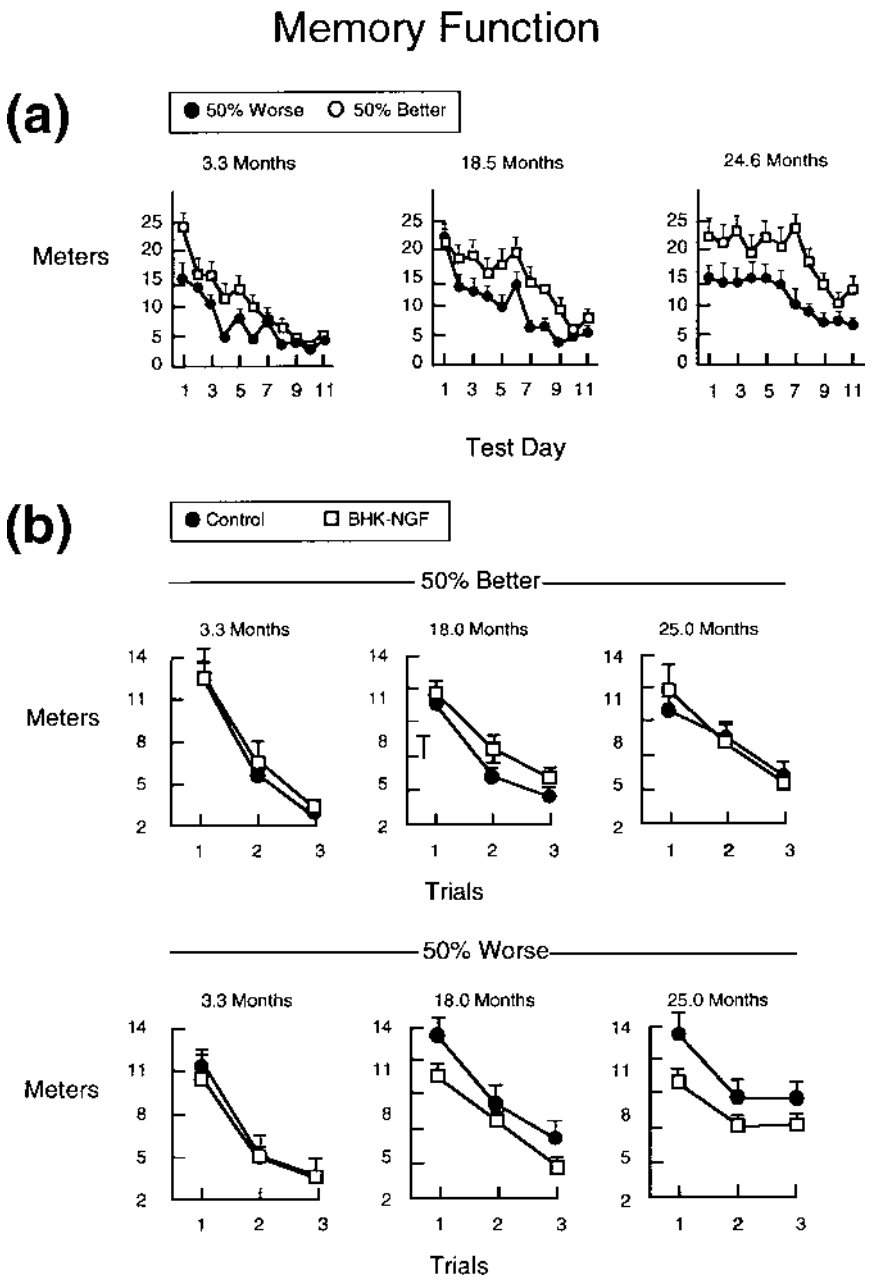

Figure 8 Cognitive function in young, middle-age, and aged rats following implantation of encapsulated NGF-producing cells into the lateral ventricles. (a) Prior to implantation, animals were divided into the 50% worse and 50% better performers in a water maze task. (b) Following implantation, NGF was found to improve performance in the middle-age and aged animals. Moreover, the improvements in cognitive performance were greatest in those animals that demonstrated the worst initial performance [see (67) for additional details].

neurons in a series of defined animal models of HD. In these experiments, rats received implants of NGF- or CNTF-producing cells into the lateral ventricles (Fig. 10). One week later, the same animals received unilateral injections of QA (225 nmol) or the saline vehicle into the ipsilateral striatum. An analysis of Nissl-stained sections demonstrated that the size of the lesion was significantly reduced in those animals receiving NGF and CNTF cells, compared with those animals receiving control implants. Moreover, both NGF and CNTF cells attenuated the extent of host neural damage produced by

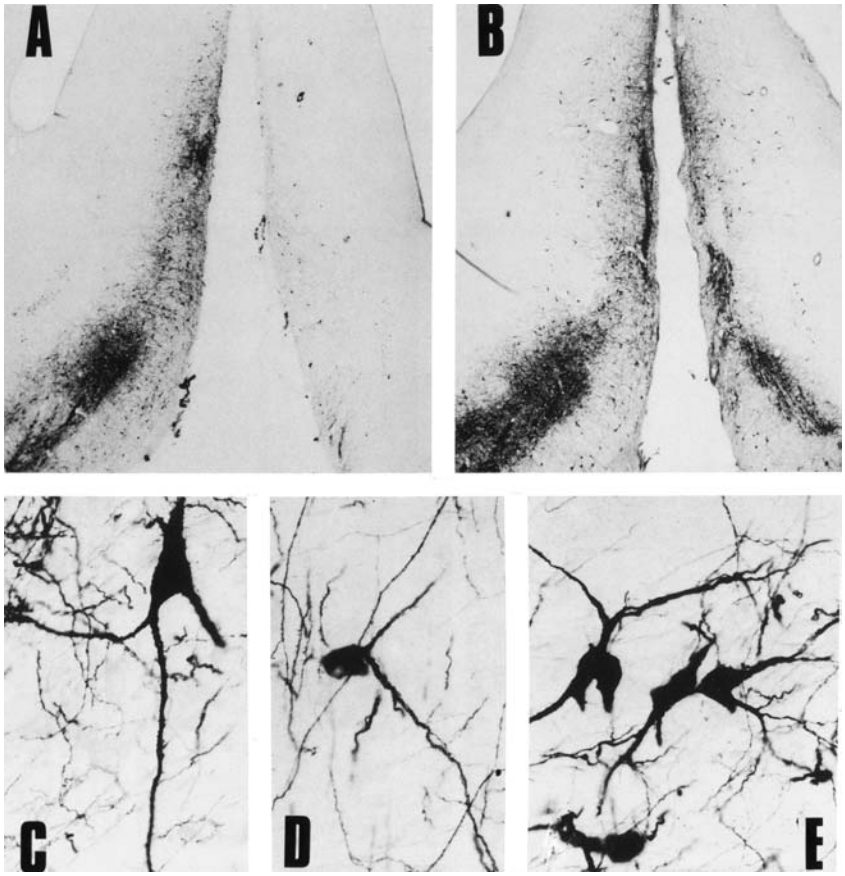

Figure 9 Photomicrographs through the septal diagonal band complex of cynomolgus monkeys. Monkeys received a fornix transection together with polymer-encapsulated implants of either BHK-control cells (A) or a BHK-NGF cells (B). Note the extensive loss of NGF receptor positive neurons ipsilateral to the lesion (right) in the control-implanted monkeys. In contrast, numerous NGF cholinergic neurons were observed in fornix-transected monkeys receiving the BHK-NGF implant. High-power photomicrographs illustrating the morphology of cholinergic neurons within the medial septum of young monkeys receiving BHK-NGF (C–E) implants. Note the enlarged perikarya and extensive neuritic arbor displayed by monkeys receiving the BHK-NGF implants [see (69) for additional details].

QA as assessed by a sparing of specific populations of striatal cells, including cholinergic, diaphorase-positive, and GABAergic neurons (Fig. 11). Neurochemical analyses have confirmed the protection of multiple striatal cell populations using this strategy (Table 3). These results suggested that implantation of polymer-encapsulated trophic factor-releasing cells can protect neurons from excitotoxin damage. Importantly, behavioral studies offer additional and compelling evidence of neuronal protection that can be produced in animal models of HD (Table 3). Trophic factor-secreting cells have provided extensive behavioral protection as measured by tests that assess both gross and subtle movement abnormalities. Moreover, these same animals show improved performance on learning and memory tasks, indicating the anatomical protection afforded by trophic factors in this model is paralleled by a robust and relevant behavioral protection (98).

B. Primate Studies

The ability of cellularly delivered trophic factors to preserve neurons within the striatum in a rodent model of HD led to similar studies in nonhuman primates, a step that is crucial to the initiation of clinical trials. A paradigm similar to the one employed in the rodent studies was used in nonhuman primates (99). Polymer capsules containing CNTF-producing cells were grafted into the striatum of rhesus monkeys. One week later, a QA injection was placed into the putamen and caudate proximal to the capsule implants. As seen in the rodent studies, the volume of striatal damage was decreased, and both GABAergic and cholinergic neurons destined to degenerate were spared in CNTF-grafted animals. Although all animals had significant lesions, there was a 3- and 7-fold increase in GABAergic neurons in the caudate and putamen, respectively,

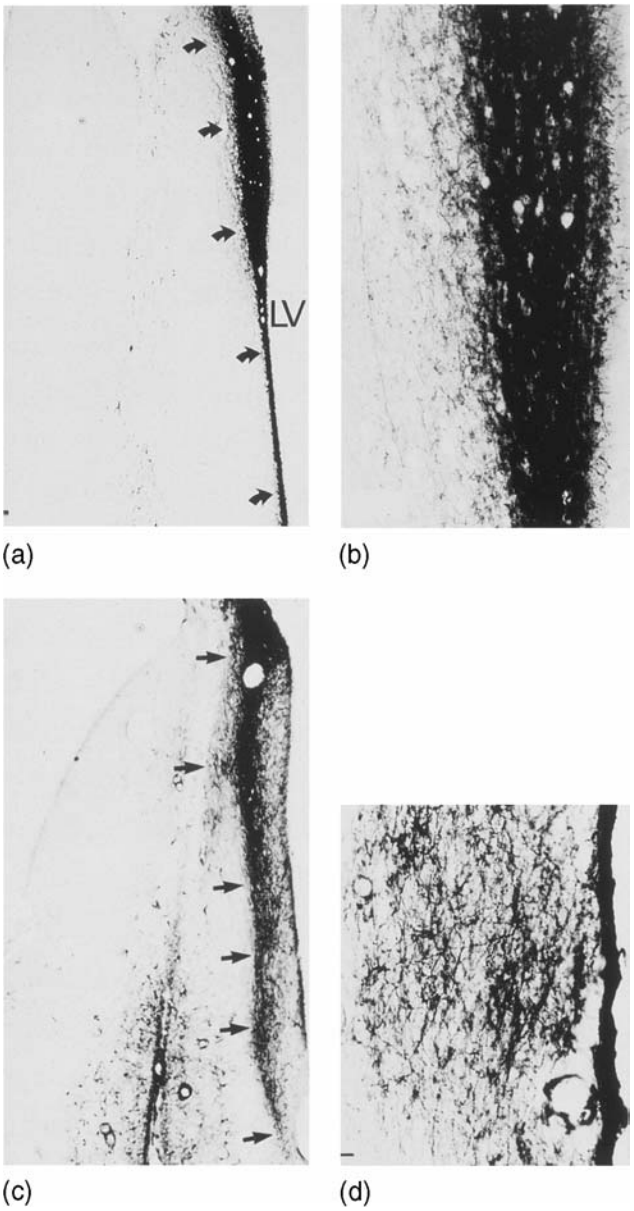

Figure 10 Sprouting of cholinergic fibers in young (a,b) and aged (c,d) NGF-treated monkeys. Low- (a,c) and high-power (b,d) photomicrographs of NGF receptor immunostained sections, illustrating a dense plexus of cholinergic fibers on the side of NGF treatment (arrows) adjacent to the lateral ventricle (LV). Scale bar in a is 1000 um for a and c; scale bar in d is 50 um for b and d.

in CNTF-grafted animals relative to controls. Similarly, there was a 2.5- and 4-fold increase in cholinergic neurons in the caudate and putamen, respectively, in CNTF-grafted animals (Fig. 12).

The ability to preserve GABAergic neurons in animals models of HD is an important, although not entirely sufficient, step to develop a useful therapeutic. If the perikarya are preserved without sustaining their innervation, then the experimental therapeutic strategy under investigation is not likely to yield significant value. The striatum is a central station in series of loop circuits that receives inputs from all the neocortex, projecting to a number of subcortical sites, and then returns information flow to the cerebral cortex. One critical part of this circuitry is the GABA-ergic projections to the globus pallidus and substantia nigra pars reticulata, the parts of the direct and indirect basal ganglia loop circuits. One approach to examining the integrity of this circuit is to use an antibody that recognizes GABA-ergic terminals (DARPP-32) to determine if the preservation of GABA-ergic somata within the striatum also results in the preservation of the axons of these neurons to critical extrastriatal sites. Using quantitative morphological assessment of DARPP-32 optical density, it has been shown that monkeys receiving QA lesions have significant reductions in DARPP-32 immunoreactivity within the globus pallidus and substantia nigra (Fig. 13). The lesion-induced decrease in GABA-ergic innervation for both of these regions was prevented in CNTF-grafted monkeys demonstrating that this treatment strategy protected GABA-ergic neurons destined to die following excitotoxic lesion, as well as sustained the normal projection systems from this critical population of neurons (99).

The intrinsic striatal cytoarchitecture can be preserved in monkeys by CNTF grafts, and once exposed to these grafts, the cells apparently maintain their projections. But are afferents to the striatum, specifically from the cerebral cortex, also influenced by these grafts? This may be particularly critical if some of the more devastating nonmotor symptoms seen in HD result from cortical changes secondary to striatal degeneration. Because layer V neurons from motor cortex send a dense projection to the postcommissural putamen, a region that was severely impacted by the QA lesion, the effects of QA lesions and CNTF implants on the number and size of cortical neurons in this region were examined. Although the QA lesion did not effect the number of neurons in this cortical area, layer V neurons were significantly reduced in cross-sectional area on the side ipsilateral to the lesion in control-grafted monkeys (Fig. 14). This atrophy of cortical neurons was virtually completely reversed by CNTF grafts (99).

A recent set of studies using CNTF-producing cells in 3NP-treated monkeys have replicated and extended these results (100). Following 10 weeks of 3NP treatment, monkeys displayed pronounced chorea and severe deficits in frontal lobe cognitive performance as assessed by the object retrieval detour test. Following implantation of CNTF-producing cells, a progressive and significant recovery of motor and cognitive recovery occurred. Histological analysis demonstrated that

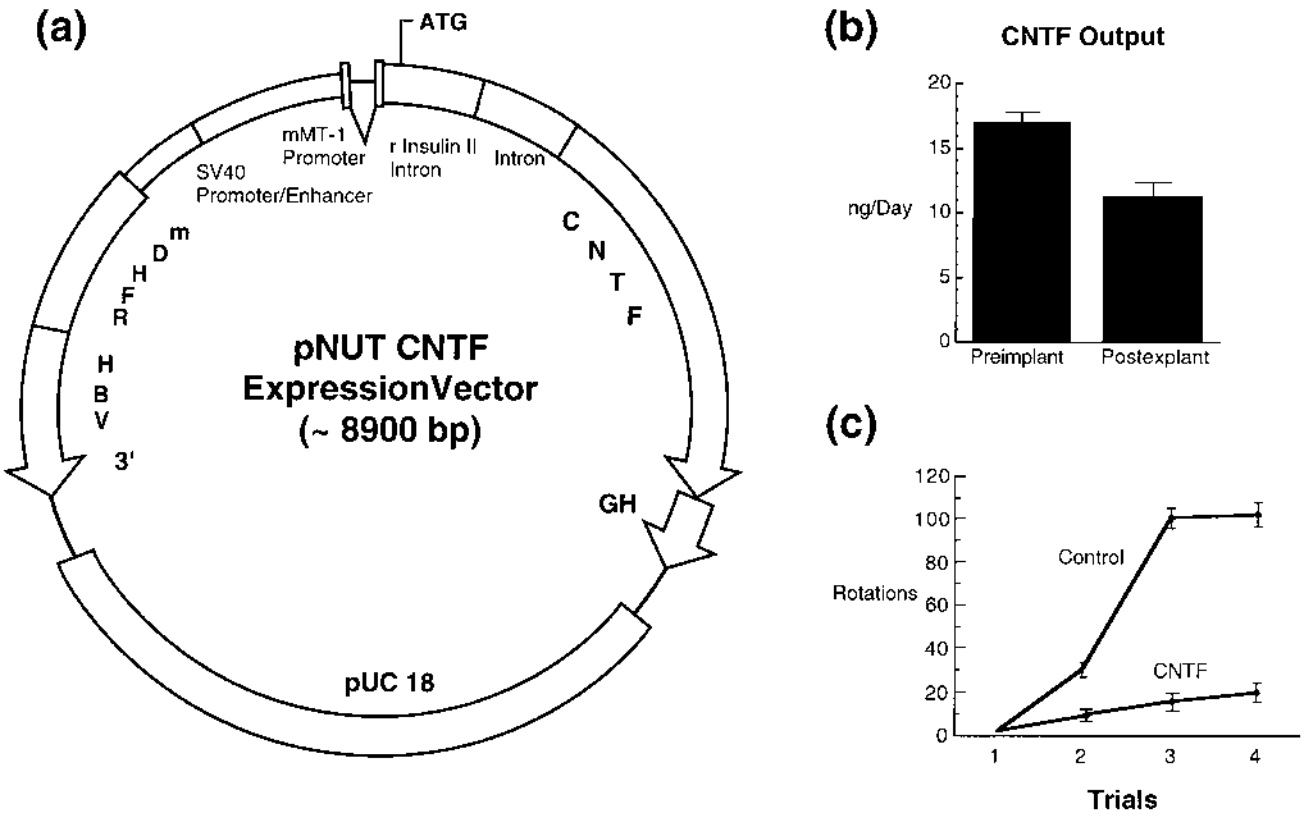

Figure 11 (a) Expression vector containing the CNTF gene. (b) CNTF levels, as determined by ELISA, in encapsulated BHK cells immediately prior to implantation (left) and immediately following retrieval from rodent lateral ventricle 70 days following implantation (right). (c) Implants of encapsulated CNTF producing cells reduce apomorphine rotations in rats after unilateral striatal injection of quinolinic acid.

CNTF was neuroprotective and spared NeuN and calbindin-positive cells in the caudate and putamen.

Although the sparing of striatal neurons and maintanence of intrinsic circuitry is impressive, the magnitude of the effect is less than that seen in rodents. In primates, robust protection is limited to the area of the capsules. However, the area of the lesion remains extensive, and it is likely that diffusion of CNTF from the capsule may not be sufficient to protect more distant striatal regions undergoing degeneration. This concept is supported by a recent experiment that examined the effects of intraventricular grafts of encapsulated CNTF grafts in the nonhuman primate model of HD (101). In contrast to when the capsules were placed directly within brain parenchyma, intraventricular placements failed to engender neuroprotection for any striatal cell types; again suggesting that diffusion is a key factor in the efficacy of this experimental therapeutic strategy. The complete lack of neuroprotection provided by intraventricular implants in primates should be considered more carefully in the current clinical trials being conducted in which encapsulated cells are being placed into the lateral ventricles of HD patients (102,103). If human trials are to yield clinically relevant positive effects, the means of CNTF delivery used in these studies needs to be improved. Whether this entails grafting more capsules, enhancing the CNTF delivery from the cells by changing the vector system or cell type employed, or changing the characteristics of the polymer membrane remains to be determined.

XIV. INITIAL CLINICAL TRIALS

A. Amyotropic Lateral Sclerosis

Neuromuscular disorders such as ALS are marked by a progressive degeneration of spinal motor neurons. The challenge for research scientists and clinicians remains to understand the etiology of this fatal disease in order to develop an effective treatment. Different families of neurotrophic factors demonstrate therapeutic potential in vitro and in animal models of motor neuron disease (104–112). The cytokine CNTF has neuroprotective effects for motor neurons in *wobbler* mice (109) and homozygote pmn (progressive motorneuropathy) mice (104,110). The delivery of CNTF to motor neurons by

Table 3 Behavioral Protection Produced by NGF and CNTF in Rodent Models of HD

Parameters measured	Neurotrophic factor	
	NGF[a]	CNTF[a]
General		
Body weight	+	+
Mortality	+	+
Motor Tests		
Receptor-activated rotations	+	+
Skilled paw use (staircase test)	−	+/−
Bracing	NE	+
Tactile adhesive removal	NE	+
Balance (rotarod)	NE	+
Locomotor activity	+	+
Haloperidol-induced catelepsy	+	NE
Cognitive Tests		
Morris water maze	NE	+
Delayed nonmatch to position operant task	NE	+

[a] NE, not evaluated; +, positive outcome; −, negative outcome.

peripheral administration proved difficult due to severe systemic side effects, short half-life of CNTF, and the inability of CNTF to cross the BBB (113–115).

Continuous intrathecal delivery of CNTF proximal to the nerve roots in the spinal cord is a practical aternative that could result in less side effects and better efficacy of CNTF in ALS patients. After safety, toxicology, and preclinical evaluation, a clinical trial to establish safety has been performed in ALS patients using polymer-encapsulated cells genetically modified to secrete CNTF (116). A total of 6 ALS patients with early stage disease indicated by a forced vital capacity greater than 75% with no other major illness or treated with any investigational drugs for ALS were included. These patients were baseline tested for Tufts Quantitative Neuromuscular Evaluation, the Norris scale; blood levels of acute reactive proteins; and CNTF levels in the serum and CSF. BHK cells were encapsulated into 5 cm long by 0.6 mm diameter hollow membranes and implanted into the lumbar intrathecal space. The device included a silicone tether, which was sutured to the lumbodorsal fascia and the skin was closed over the device (Fig. 15). CNTF concentrations in the CSF were not detectable prior to implantation, but was found in all 6 patients at 3 to 4 months postimplantation. All 6 explanted devices had viable cells and CNTF secretion of approximately 0.2 to 0.9 μg/day. No CNTF was detected in the serum.

B. Chronic Pain

Numerous studies with rodent models of acute and chronic pain have suggested that adrenal chromaffin cells implanted into the intrathecal space and in the periaqueductal gray reliably produce significant analgesic effects [see (117,118) for a review]. Although the majority of these studies have used unencapsulated cells, recent studies suggested that encapsulated cell implants also produce analgesia in rats. The analgesic effects of adrenal chromaffin cells in rodent model have provided the rationale to pursue clinical trials in patients with chronic pain. Small, open-label trials demonstrated that the implantation procedure was minimally invasive and well tolerated (119,120). Neurochemical and histological studies determined that the encapsulated cells survived and were biochemically functional for up to 1 year. Because reductions in morphine intake were noted following implantation (suggesting efficacy), larger-scale, randomized studies were initiated in a collaborative study between CytoTherapeutics and Astra Pharmacueticals. Unfortunately, the trials were recently halted because the efficacy achieved did not reach a level high enough to warrant further study (121).

Interestingly, several recent reports indicate encapsulated adrenal chromaffin cell implants may not produce efficacy as originally suggested (122–125). Extensive studies in acute and chronic rodent pain models have failed to find any evidence of analgesia. This lack of effect occurred under conditions that were apparently designed to exactly reproduce previous testing procedures that did demonstrate efficacy. Among the variables examined were the location of implant (intrathecal vs. intraventricular), a wide range in cell preparation techniques, and an exhaustive battery of acute and chronic pain tests with and without nicotine stimulation. Importantly, the authors reported that systemic administration of morphine produced significant analgesia when tested in parallel in the same models. Although subtle testing differences cannot be ruled out as contributing factors in the differences between these recent and previous studies, together with the only well-controlled clinical trial conducted to date, it appears that, at the least, adrenal chromaffin cells do not produce analgesic effects as consistently as previous reported (122–124).

C. Huntington's Disease

Recently, clinical trials were initiated to determine the safety and tolerability of CNTF-producing cells implanted into the lateral ventricle of HD patients (37). Although the case for clinical evaluation is compelling, several issues are apparent with the design of the ongoing clinical trials. Although the sparing of striatal neurons and maintanence of intrinsic circuitry in monkeys is impressive, the magnitude of the effect is less than that seen in rodents. Robust protection is largely limited to the area of the capsules. However, the area of the lesion remains extensive, and it is likely that diffusion of CNTF from the capsule may not be sufficient to protect more distant striatal regions undergoing degeneration. This concept is supported by a recent experiment that examined the effects of intraventricular grafts of encapsulated CNTF grafts in the QA monkey model of HD. In contrast to when the capsules were placed directly within brain parenchyma, intraventricular placements failed to engender neuroprotection for any striatal cell types; again suggesting that diffusion is a key factor in

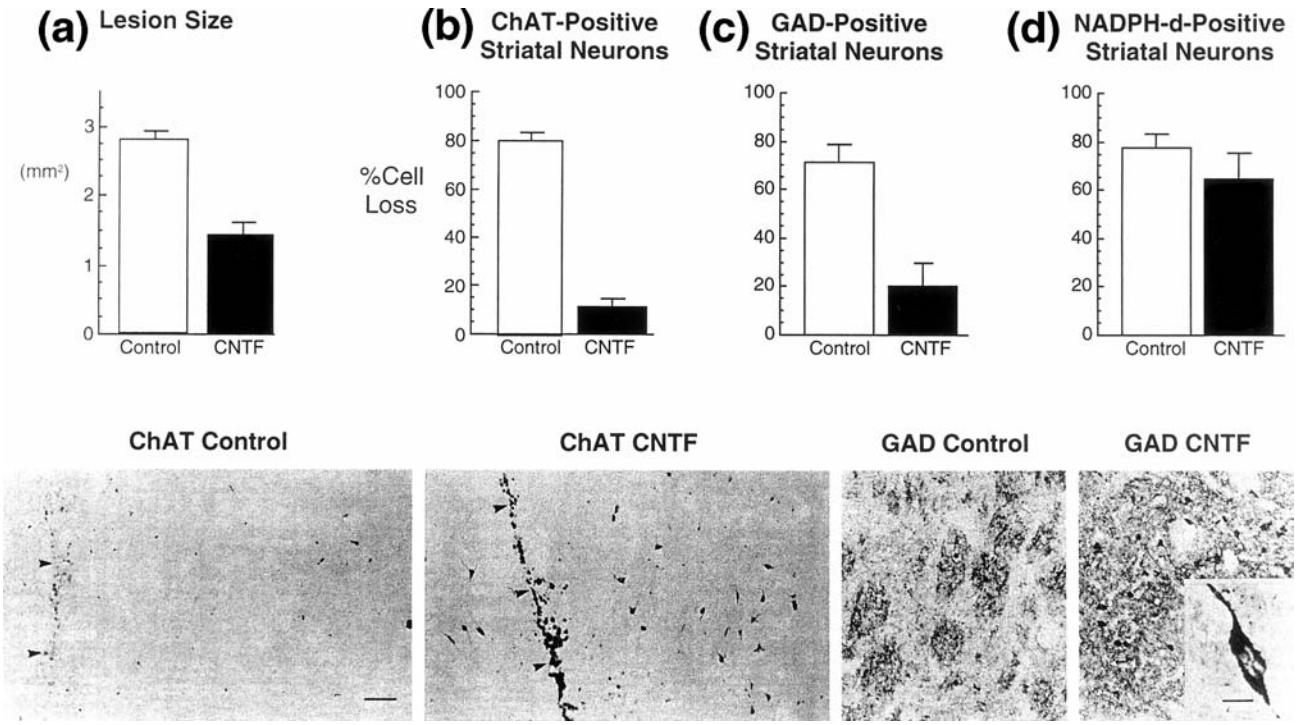

Figure 12 Lesion volume and neuronal cell counts in quinolinic acid-lesioned rats. Control-implanted animals displayed a marked lesion volume (a, determined by Nissl staining) and a significant loss of multiple types of striatal cell types, including cholinergic (b), GABAergic (c), and diaphorase-positive neurons (d). The cholinergic and GABAergic neuronal losses were largely prevented in animals receiving CNTF implants, whereas the loss of diaphorase-positive neurons was not affected. In each case, data are presented as a percent loss of neurons on the lesioned/implanted side compared with intact contralateral side. Representative photomicrographs for cholinergic and GABAergic cells are shown for both control and CNTF-implanted animals. Note the appearance of numerous healthy appearing cholinergic and GABAergic neurons in the CNTF-treated animals. Scale bar in ChAT control is 500 um for ChAT control/CNTF, and scale bar in insert is 100 um for GAD control/CNTF and 17 um for insert [see (97) for additional details].

the efficacy of this experimental therapeutic strategy (101). The complete lack of neuroprotection provided by intraventricular implants in monkeys should be considered more carefully in the ongoing clinical trials (38). If human trials are to yield clinically relevant positive effects, the means of CNTF delivery used in these studies needs to be improved. Whether this entails changing the site of implantation from the ventricle to the parenchyma, grafting more capsules, enhancing the CNTF delivery from the cells by changing the vector system or cell type employed, or changing the characteristics of the polymer membrane remains to be determined.

XV. CONCLUSIONS AND FUTURE DIRECTIONS

Since the late 1980s to early 1990s, a substantial body of data has been collected, clearly showing the promise of encapsulated cell therapy for treating a wide range of CNS disorders. Still, a number of research avenues exist that are incompletely explored and deserve attention prior to wide-scale clinical use of this technology. In some preclinical studies, the extent of diffusion from the implants appears to limit the therapeutic effectiveness of the encapsulated cells (62,68,69). Given the size of the human brain relative to the rodent and nonhuman primate brain, the potential problems related to limited tissue diffusion should be examined empirically. Studies in larger animals, such as nonhuman primates, should be conducted to include considerations of different numbers of devices and multiple reimplants distributed over long periods of time (>12 months). In this way, a reasonable assessment of the optimal spacing and distribution of multiple implants can be determined. This information could, at the same time, provide critical information regarding the relative risks of repeated tissue penetrations, tissue damage, and the potential for infection.

Encapsulation provides the opportunity to use cells from a variety of sources, including human and animal sources with and without genetic modification. In theory, the capsule should isolate the cells from the surrounding tissue. Still, if a capsule ruptures during implantation or retrieval, a deleterious

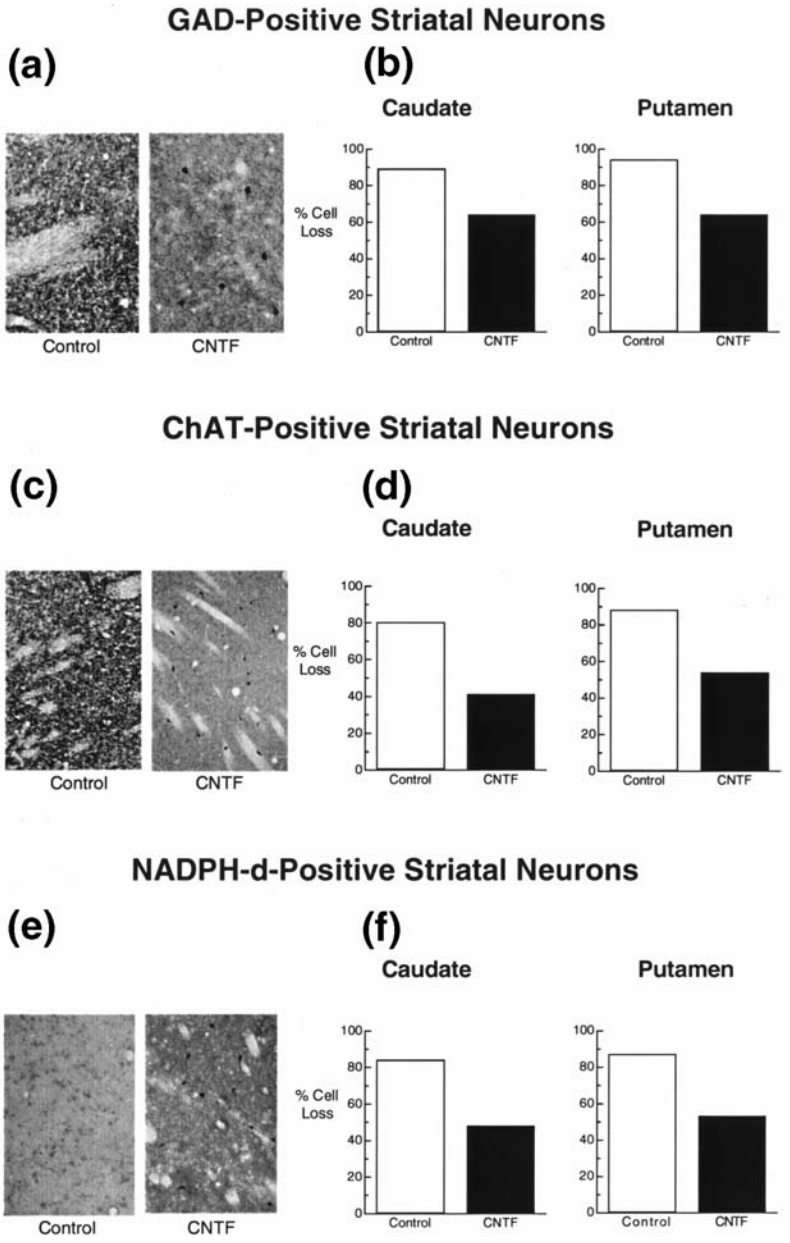

Figure 13 Neuronal cell counts in quinolinic acid-lesioned monkeys. Control-implanted animals displayed a significant loss of multiple types of striatal neurons including GABAergic (a and b), cholinergic (c and d), and diaphorase-positive neurons (e and f). Although neuronal loss was still present in animals receiving CNTF implants, it was significantly attenuated in both the caudate and putamen. In each case, data are presented as a percent of neurons on the lesioned/implanted side compared with the intact contralateral side. Representative photomicrographs for all 3 cell types are shown for both control and CNTF-implanted animals (a, GABAergic; c, cholinergic; and e, diaphorase-positive neurons) [see (99) for additional details].

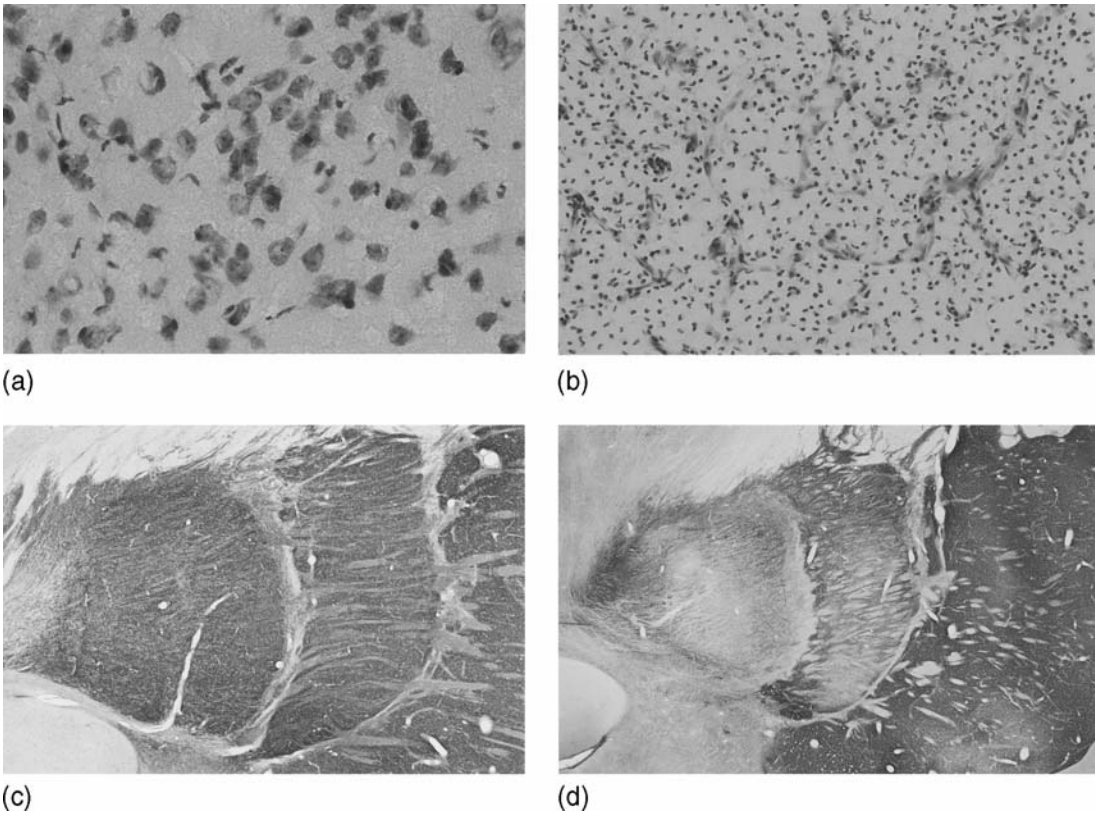

Figure 14 Photomicrographs of Nissl-stained sections through the striatum of monkeys that received quinolinic acid injections into the striatum followed by implants of encapsulated CNTF-producing (a) or control (b) BHK cells. A paucity of healthy neurons is observed in the striatum of control monkeys, which is in stark contrast to the numerous healthy appearing neurons seen in the same region of the CNTF-treated monkeys. Together with the sparing of strital neurons is a preservation of the GABE-ergic projection from the striatum to the globus pallidus. DARPP-32 immunocytochemistry revealed an intense, normal-appearing immunoreactivity within both the external and internal segments of the globus pallidus of CNTF-treated animals (c). In contrast, DARPP-32 immunoreactivity is reduced in control-implanted animals as a consequence of the lesion (d). Quantitative analysis confirmed the sparing of this projection in CNTF-treated monkeys.

host immunological response could be induced. Although the host immune system should reject any released cells following capsule damage, the potential for tumorous growth remains a safety concern. Alterations in the ability of the host immune system to reject cells following damage to implants could also change upon long-term residence of the cells within the host. To date, no studies have systematically evaluated these risks, particularly with regard to the long-term effects of encapsulated cell implants. Again, primate studies using intact and intentionally damaged devices would provide a useful starting point for evaluating these issues. These studies could use normal and immunosuppressed animals, as well as evaluate potential tumorigenicity and changes in the host immune system over short and long periods of time.

Regulation of dosage is another area that deserves attention. In its most basic iteration, varying the numbers of cells within an implant, the size of the implant, or the use of multiple implants, may permit a range of doses to be delivered. Although some long-term cell survival studies have been conducted (61–65), they have not systematically examined cell survival and output of the desired molecule over long periods of time. Rather, studies have provided a ''snapshot'' of survival and output at a single timepoint. Large, long-term, well-controlled studies need to be conducted to examine the relationship between variables that include time, cell survival, gene expression (when modified cells are used), neurochemical output, the initial numbers of cell encapsulated, and the type of semipermeable membrane and extracellular matrix used for encapsulation. Obviously, such studies are time consuming and expensive. However, without them, the conditions optimal for successful cell encapsulation will remain speculative. It should be pointed out that some efforts are ongoing in this area, and a recent study raised the interesting possibility that dose control for dividing cells could be accomplished

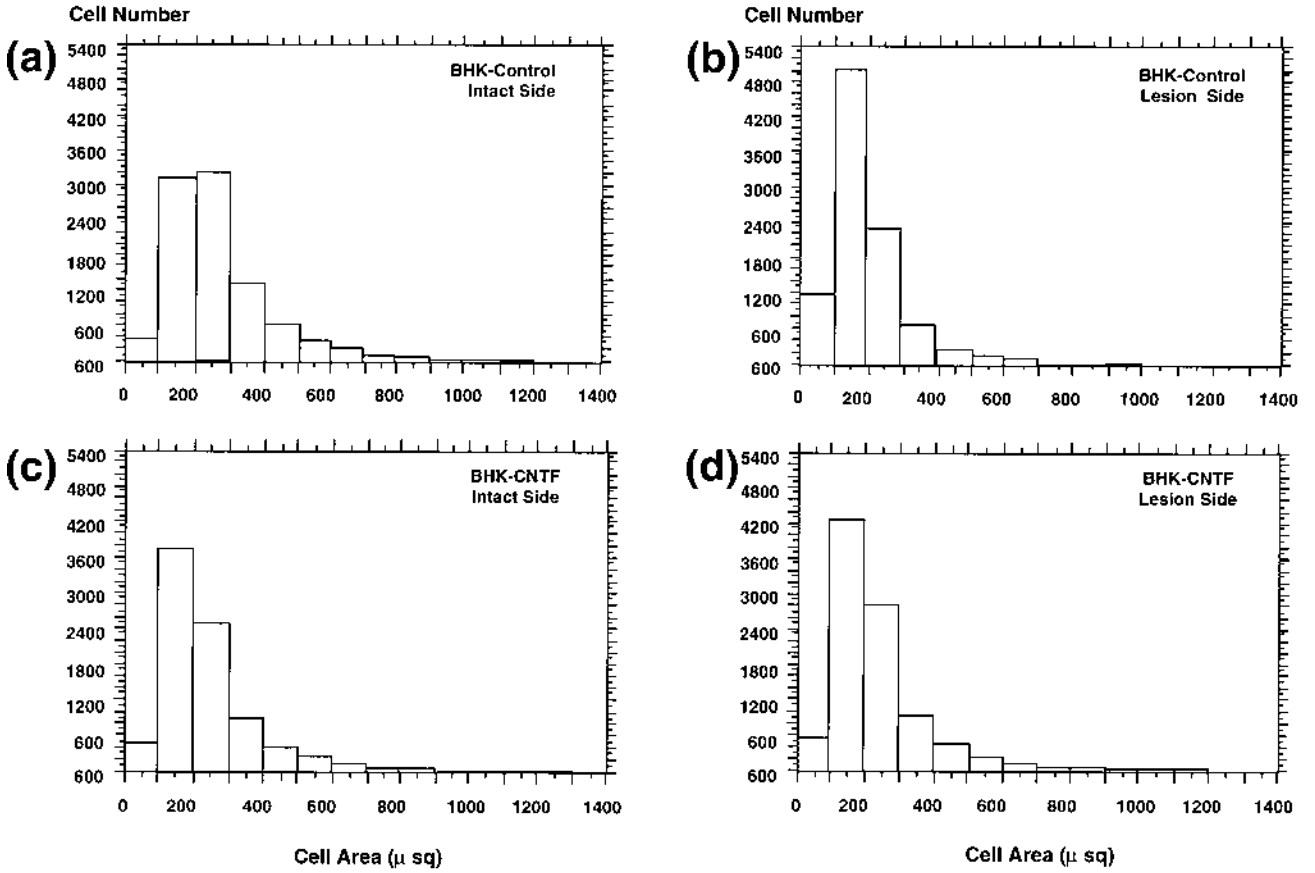

Figure 15 The cells size distribution of layer V neurons in the motor cortex of monkeys that received striatal injections of quinolinic acid followed by implants of encapsulated BHK control (a,b) or CNTF-secreting BHK cells (c,d). This figure demonstrates that CNTF-producing grafts prevented the atrophy of cortical neurons that innervate the striatum. Note that in the control animals there is an increase in the number of neurons in the 0 to 100 μm and 100 to 200 μm range and a decrease in the number of neurons in the 300 to 400 and 400 to 500 μm range ipsilateral to the lesion. This shift in cells size was not seen in those animals receiving CNTF-secreting cells.

with the use of cell-containing microcarriers in nonmitotic hydrogels (126).

Another area that has attracted little attention, concerns the variability in the in vivo performance of encapsulated cells and the possible role that the host tissue environment plays in this variability (51,66,67). As discussed earlier, it appears that at least some of the variability in device performance is attributable to differences between hosts. Although the mechanism(s) underlying these individual differences remain undetermined, several potential candidates exist, including the variations in the general health of the animals, between animal differences in immune function and undetected microbreaches in the polymer membrane prior to or during implantation. The notion that the viability of grafted cells may depend in part on host-related variability in the CNS environment has only been suggested for encapsulated cells to date. However, this emerging concept might also prove to be relevant for all CNS transplantation approaches that are cellular based. Indeed, the entire field of neural transplantation might benefit from this new perspective uncovered using encapsulated cells.

Finally, very few clinical studies have been conducted to date. Although several small safety studies have been completed, only one large, controlled clinical study has been performed using encapsulation technology. This study evaluated the use of encapsulated adrenal chromaffin cells for the treatment of pain but failed to reveal analgesia sufficient enough to continue the trials. As we have already discussed in a previous section, the selection of pain as an initial indication for detailed study might have been an unfortunate choice given that recent preclinical data using encapsulated chromaffin cells is mixed at best. The only other clinical targets under investigstion are ALS and HD, and these are apparently modest efforts. Until, larger, controlled clinical trials are conducted, the potential of this technology will not be fully realized.

In conclusion, it appears that the implantation of encapsulated cells may provide an effective means of alleviating the

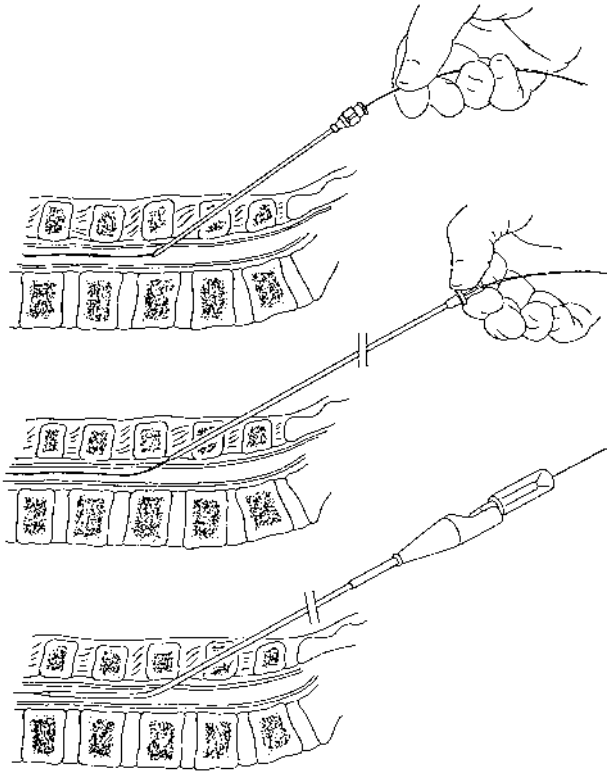

Figure 16 Under local anesthesia, a device containing CNTF-secreting cells is implanted into the lumbar subarachnoid space via a small incision over the lumbar spine. A 19-gauge Touhy needle is inserted into the subarachnoid space. A flexible tip wire is inserted, the needle withdrawn, and a dilator passed through. A smaller cannula is then inserted over the wire and the cell-containing device is guided through the subarachnoid cannula. The silicone tether is secured to the lumbar fascia and the skin is sutured closed over the entire device.

symptoms of numerous human conditions/diseases. One particularly attractive avenue of research continues to be the application of trophic factors to minimize or halt the progression of neural degeneration or promote regeneration of damaged central nerves. However, caution must be applied when considering any novel therapy for treating brain disorders, and the wide-scale use of polymer neural implants should be considered only after rigorous scientific experimentation in animal models and their demonstrated efficacy and safety in human clinical trials.

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43

Hematopoietic Progenitor Cells

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I. INTRODUCTION TO HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSCs) have been a focus of interest in the field of gene therapy since its inception, due to their 2 defining characteristics: their ability to differentiate into all the component cells of the hematopoietic system, and their ability to renew themselves in vivo. Genetic modification of the HSCs should result in modified cells of all lineages, including erythrocytes, platelets, myeloid cells, and lymphocytes. Due to the self-renewal properties of stem cells, genetic modification would be, at least in theory, very permanent. Despite great expectations for HSC gene therapy, clinical application has been slow primarily due to the inefficiency of gene transfer into HSCs (see Chapter 24). In addition, an inability to purify and expand these cells in vitro has hampered the development of improved transduction protocols. Finally, some of the diseases initially considered for HSC gene therapy, such as sickle-cell anemia, proved to be ambitious targets due to the complex regulation of gene expression required (see Chapter 25). Nevertheless, improvements in our understanding of the biology of HSC are being made, which will ultimately make HSC gene therapy more widely applicable, potentially even to diseases outside the hematopoietic system. In this chapter, we review the basic biology of murine and hematopoietic stem cells, explore a variety of genetic transduction strategies, and briefly review recent clinical attempts and HSC gene therapy.

II. PHENOTYPE OF HSC

A. Cell Surface Phenotype of Murine and Human HSC

The existence of the HSC was first suggested by experiments showing rescue of mice from irradiation by infusion with non-irradiated whole bone marrow (WBM) (1). Because WBM is a heterogeneous mixture of cells, identification of the cells responsible for the radiation rescue has been a focus of interest for some time. Physical properties, such as sedimentation rates, of bone marrow (BM) cell subsets were initially exploited, followed by antigenic or dye staining properties that could be identified by fluorescence-activated cell sorting (FACS).

Class I major histocompatibility antigens (MHC I) were one of the first molecules exploited for fractionating WBM (2). Depletion of activity assays, which used antibodies capable of fixing complement and directing the lysis of target cells, were employed to show that murine HSC did not express MHC I (2). In contrast, human primitive progenitors do express MHC I (3), establishing the first of many phenotypic differences between murine and human HSCs. Ultimately, it was established that that murine progenitors and HSC do not express MHC I, while human progenitors express high levels of MHC I, and human HSCs express low levels (4–7).

Cell ''panning'' has also been employed to fractionate WBM, where an antibody is fixed to the surface of a tissue culture plate and BM cells, which adhere to the antibodies, are collected and assayed for the enrichment or depletion of primitive hematopoietic potential. Cell panning helped identify c-kit (8) expression by HSCs as well as their ability to bind wheat germ agglutinin (9).

The development of flow cytometry allowed multiple antigens or fluorescent dye efflux characteristics to be used together for improved purification. A major advance was to use a cocktail of antibodies against antigens specific for the differentiated lineages to deplete the BM of the 90% differentiated cells (10). Such ''lin''—negative cells in human or mouse BM is depleted of CD4, CD8, and CD3 T cells, B cells, granulocytes, macrophages, and erythrocyte progenitors. Antibodies against human CD33 are used, as it is ex-

pressed by progenitors but not HSCs (11). Similarly, CD38 is present on human progenitors but absent from HSCs (12), so can be used for negative selection of differentiated cells.

Another major breakthrough in HSC purification entailed the use of the CD34 antigen for positive selection of human HSCs (6,13). Primate studies ushered in the use of CD34 as a tool for isolating HSCs for bone marrow transplantation (14), and CD34 became the most clinically exploited molecule expressed by HSC.

The Thy-1 antigen has also been an important tool for enrichment of murine and human HSC (15), as has been Sca-1 (for stem cell antigen) (10). Although Sca-1 is routinely used to purify murine HSCs, neither a human homolog nor function have been identified for this molecule. c-kit expression has also been used to purify both human and murine HSCs (16–20). $Lin^{Neg}c\text{-}kit^{Pos}$ murine WBM cells can repopulate mice ablated by irradiation (21).

In summary, murine and human HSCs can be selected on the basis of the expression of a number of antigens in combination: Sca-1, Thy-1 (low), $lineage^{neg}$ $c\text{-}Kit^{pos}$ in the mouse, and CD34, Thy-1 (low), $lineage^{negative}$, and $c\text{-}kit^{pos}$ in the human.

B. Markers That Change as HSCs Mature

Although CD34 clearly marks human HSCs, the most primitive murine HSCs are $CD34^{Neg}$ (22,23) and are precursors of $CD34^{Pos}$ short-term HSCs (24). When the gene for human CD34 was introduced into mice, HSCs that were negative for murine CD34 expressed the human CD34 antigen (25), suggesting that $CD34^{Pos}$ human HSCs are analogous to $CD34^{Neg}$ murine HSCs and that CD34 expression is differentially regulated in mice and humans (Fig. 1).

HSCs also vary cell surface receptor expression as they progress in the cell cycle. 5-Fluorouracil (5-FU) treatment ablates progenitors and proliferating cells in the bone marrow (26), and induces quiescent stem cells to proliferate and replenish the bone marrow. 5-FU treatment/cell-cycle induction concomitantly induces changes in several surface receptors: c-kit is down-regulated and CD34 is up-regulated on murine HSCs (24,27). Furthermore, in treated mice, both $CD34^{Pos}$ and $CD34^{Neg}$ cells are capable of long-term WBM repopulation—in untreated mice, only $CD34^{Neg}$ cells have this activity. Therefore, murine $CD34^{Pos}$ HSCs may be newly cycling HSCs.

Some cell surface markers are developmentally regulated on HSCs. AA4.1 was identified on fetal liver HSCs and HSCs isolated from embryonic yolk sac (28), but not adult HSCs. Also, Sca-1, expressed by adult HSCs, is not expressed by yolk sac HSCs (29), but is up-regulated on fetal liver HSCs (30,31). AA4.1 is expressed on 5-FU-treated HSCs, potentially correlating with their actively cycling status, as in the fetal liver (32).

C. Vital Dye Enrichments

Vital dyes have also been used alone or in combination with cell surface markers. Most WBM cells stain brightly with the DNA-binding dye Hoechst 33342, but progenitors stain dimly (33–35). Hoechst-based enrichment for stem cells was refined with the observation that with the use of 2 emission wavelengths, multiple populations could be defined. A small population can be observed on the side of the profile [the so-called side population (SP)] (Fig. 2) (36), which contains all the hematopoietic reconstituting potential of mouse bone marrow. An SP has been observed in multiple species (23) and appears to be due to a multidrug resistance (MDR)-like mediated efflux of the Hoechst dye (36). The efflux of Hoechst dye by HSCs is believed to be due to the transporter ABCG2 (37), but additional drug pumps are likely also involved. Although the natural substrate of the dye efflux is currently unknown, we hypothesize that it may be a molecule that otherwise would cause the cells to differentiate. FACS purification using the SP profile alone affords an enrichment of 1000- to 3000-fold. When used in combination with additional cell surface markers, another 15% or so of nonstem cells can be eliminated (23,36,38).

Rhodamine 123 (Rh123) is another vital dye used for HSC enrichment. Murine and human $rhodamine^{Low}$ WBM cells are enriched for HSCs (39,40). When $Hoechst^{Low}$ WBM cells were fractionated into $rhodamine^{Medium}$ and $rhodamine^{Low}$ populations (35), $Rhodamine^{Low}$ cells were shown to be more primitive (35). Rh123 binds mitochondria, and therefore Rh123 fluorescence is believed to directly reflect cellular metabolic activity (41). However, because Rh123 is also a substrate for MDR transporters, low Rh123 may simply reflect dye efflux, as is the case with Hoechst 33342.

D. Phenotype of HSC from Different Sources

HSC can be derived from other sources besides WBM, such as peripheral blood (PB), fetal cord blood (CB), fetal liver, skeletal muscle, and embryonic stem cells, with some phenotypic variations. In 1951, Brechen et. al. found mice were rescued from irradiation by parabiosis with nonirradiated partners (42), suggesting that HSCs can circulate in PB. Infusion with >5 million nonirradiated PB cells also rescued irradiated mice, showing HSCs were present in the circulation at very low frequencies (43). Human PB also contains cells with primitive hematopoietic potential (44,45), although these cells are infrequent and less potent than WBM-derived HSCs (46,47). Mobilization with GM-CSF can greatly increase the frequency of HSCs in human PB (48) and is a common strategy for enriching and purifying HSCs from PB (49).

PB-derived HSCs are phenotypically distinct from WBM HSC. Although both human WBM and PB HSCs express CD34 and are $Rhodamine^{Low}$ (50,51), mobilized PB HSCs express lower levels of c-kit than WBM HSCs (52).

HSCs are also present in newborn CB. Hematopoietic progenitors in CB have been shown to express CD34 both in vitro and in vivo (53–56). HSCs in CB are resilient to cryopreservation and extensive handling and are therefore a clinically useful source of HSCs (57,58). CB HSCs are phenotypi-

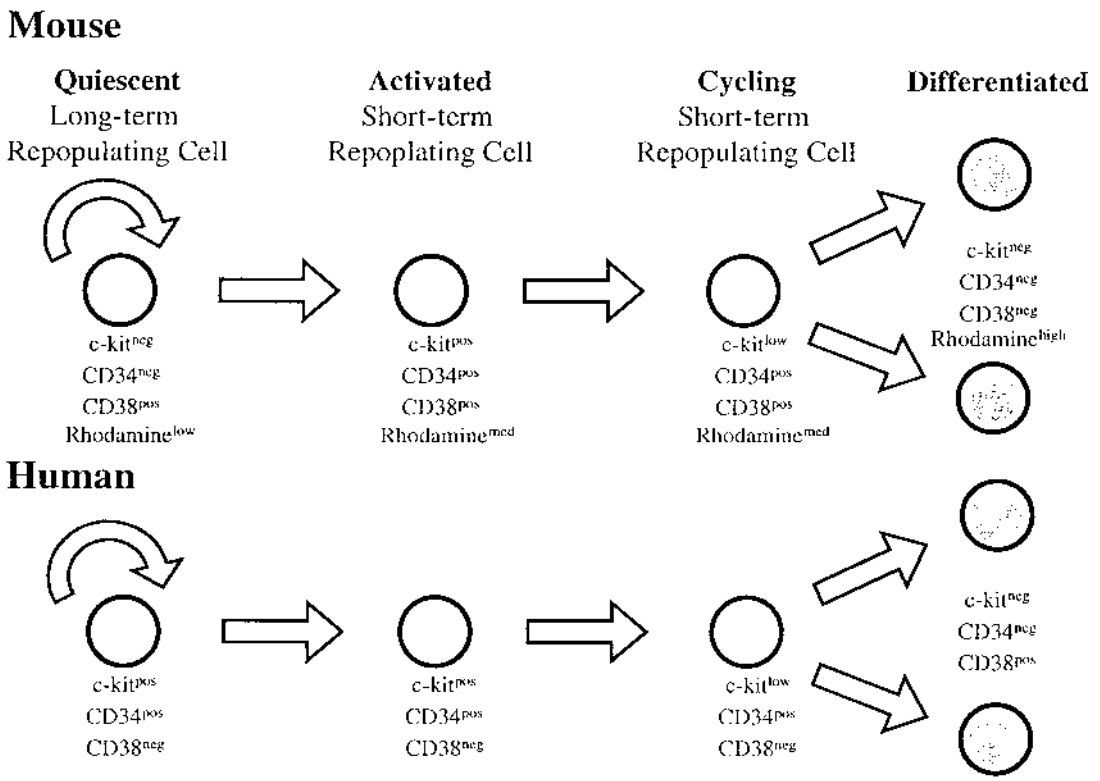

Figure 1 Progression of cell surface marker expression on maturing murine and human HSC. Summary of cell surface markers that distinguish quiescent, activated, newly cycling, and differentiating HSCs in humans and mice.

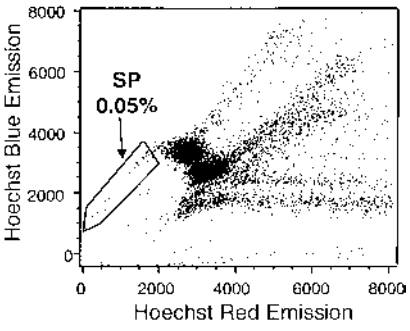

Figure 2 Representative Hoechst 33342 staining profile of murine bone marrow. When mouse bone marrow is stained with the DNA-binding dye Hoechst 33342, multiple populations are observed. All the hematopoietic reconstituting cells are found in the small population on the side, the ''side population'' or SP (boxed). See references for staining protocol.

cally similar to WBM HSCs, although there is controversy in the literature regarding their expression of CD38 (59,60).

During development, HSCs initially appear in the aortes-gonad-mesonephres (AGM). They subsequently migrate to the fetal liver and ultimately to the fetal bone marrow. As previously mentioned, AA4.1 is specifically expressed by fetal liver-derived HSCs in mice (28,31,61). HSCs isolated from the AGM and fetal liver of mice are Sca-1^{Pos}c-kit^{Pos} CD34^{Pos}Lin^{Neg}WGA^{Pos}CD43^{Pos} and Rhodamine^{Bright} (30,62). Thus, in contrast to murine WBM HSCs, embryonic HSCs take up high levels of rhodamine and express CD34, perhaps suggesting they are more activated than adult HSCs. Human fetal liver HSCs have been characterized as CD34^{Pos}CD38^{Neg} CD33^{Pos}Lin^{Neg} (63). Thus, they also differ from human WBM HSCs in their expression of CD33.

Embryonic stem (ES) cells can differentiate into hematopoietic cells. When ES cells are cultured into embryoid bodies, blood islands containing erythrocytes and macrophages spontaneously emerge (64,65). Lymphoid, myeloid, and erythroid colonies can also be found to develop (66). ES cells can be directly induced to differentiate into myeloid and B cells by eliminating M-CSF from culture medium (67). Human ES cells up-regulate CD45 expression, a hematopoietic marker,

when cultured on methylcellulose containing hematopoietic cytokines (68). ES cells isolated from rhesus monkeys develop into clusters of hematopoietic cells and up-regulate both CD34 and several hematopoietically relevant genes when cultured on S17 feeder cells along with stem cell factor, interleukin-3 (IL-3), IL-6, vascular endothelial growth factor, and G-CSF (69). In summary, ES cells can be induced to behave like HSCs in vitro under specific culture conditions, although these cultures are still not well defined.

ES cells can also function as HSC in vivo. Three-day-old blastocysts were found to rescue mice from lethal irradiation (70), and desegregated embryoid bodies were reported to moderately restore the lymphoid system of severe combined immune deficiency (SCID) mice (71). Transduction of ES cells with hematopoietic transcription factors, such as BCR/Abl and HoxB4, induced their differentiation into HSC and allowed for engraftment of the myeloid and lymphoid lineages after transplantation into irradiated mice (72,73). However, the engraftment was skewed toward the myeloid lineages. Thus, the ability to direct the differentiation of ES cells into HSCs is still limited and inefficient.

Finally, cells with HSC activity, initially believed to be trans-differentiating muscle stem cells, can also be isolated from the skeletal muscle of mice (74,75). Muscle-derived HSCs express both CD45 and Sca-1 and are therefore hematopoietic in origin (76,77). Muscle-derived HSCs have since been shown to fall into the SP compartment of skeletal muscle and be c-kit^{Dim}, relative to WBM HSCs (McKinney-Freeman, Experimental Hematology in press). Muscle-derived HSCs isolated from neonatal mice are reportedly CD45^{Neg} (78). If a nonhematopoietic HSC does exist in neonatal murine muscle, it is clear that this cell population does not persist in the adult animal (76,77) (McKinney-Freeman, Experimental Hematology in press).

III. IN VIVO AND IN VITRO ASSAYS FOR ASSESSING HSC FUNCTION

The discovery that nonirradiated WBM rescued mice from irradiation (1) established the existence of the HSCs, but was unable to measure HSC reconstituting activity because WBM is a heterogeneous mixture of HSCs and progenitors. Below is a discussion of the development of in vitro and in vivo assays for assessing and quantitating HSC function and differentiation.

A. CFU-S and CFU-C Assays

In 1961, Till and McCulloch observed macroscopic colonies of hematopoietic cells in the spleens of mice 8 days after injected with WBM (79). They dubbed the colonies ''colony forming units'' (CFU), observed that the frequency of colony formation was proportional to the number of injected cells, and concluded that spleen colony formation quantitatively reflected HSC activity. The demonstration that CFU-spleen (CFU-S) were capable of self-renewal, solidified this conclusion (80). Thus, Till and McCulloch developed the first quantitative assay of primitive hematopoietic potential.

The CFU-S assay was accepted as a method for assessing HSC potential. However, CFU-S activity was found to be sensitive to 5-FU treatment (81), with CFU-S_8 more sensitive than those appearing on days 12 to 14 (CFU-S_{12-14}) (82). HSCs are quiescent, and therefore resistant to the effects of 5-FU. It was concluded that the CFU-S assay actually measured WBM progenitor activity, with CFU-S_{12-14} reflecting the activity of more primitive progenitors than CFU-S_8, and that true HSCs may be ''pre-CFU-S'' cells (81).

In vitro assays were later developed to test the role of specific factors in hematopoiesis and evaluate the hematopoietic potential of human cells. Tightly packed, clonal colonies of differentiating cells form when WBM is overlaid on embryonic feeder cells in agar (83,84) or grown with appropriate growth factors (Fig. 3). The feeder cells supplement the agar with hematopoiesis-promoting factors, and the result is colonies of granulocytes and macrophages, called CFU-G and CFU-M, respectively (83). Primitive colonies of mixed cells derived from multipotent progenitors also form and are called CFU-GM and CFU-GEMM, for granulocyte, erythrocyte, monocyte, and macrophage (85). Erythropoietin promotes the development of erythrocytic colonies (86,87). Methylcellulose can be used as an alternative semisolid medium (88) and infusing the medium with fetal calf or human serum eliminates the need for feeder cells (85).

The in vitro CFU-culture (CFU-C) assay was initially believed to measure the activity of the same cell that forms CFU-S because erythrocytic CFU-C can form CFU-S when injected into mice (89). However, CFU-S and CFU-C activity can be separated by size and density (88) and may actually reflect the activity of distinct progenitors. Regardless, both the in vivo CFU-S and in vitro CFU-C assay measure hematopoietic progenitor activity.

The LTC-IC (long-term culture initiating cell) assay was developed to measure primitive (pre-CFU-C) human hematopoietic progenitor activity in vitro. In this assay, cells are cultured on a feeder layer of irradiated human bone marrow-derived stroma cells and then assayed for CFU-C activity 5 to 8 weeks postplating in order to extrapolate the number of LTC-IC present at initial plating (6). Figure 4 summarizes the primitive hematopoietic populations assayed by the different in vitro assays discussed above.

B. In Vivo Models

The ability of HSC to maintain lifelong hematopoiesis can only be assayed in vivo. Several animal models have been developed for assaying both murine and human HSC activity in vivo.

W/W mice have a spontaneous mutation in their c-kit receptor and die shortly after birth due to anemia and cytopenia unless rescued by transplantation with normal WBM (90). Thus, the systemic deleterious effects of lethal irradiation are avoided by using these mice as an in vivo model. W/W mice have been used to show that fetal liver contains HSCs capable of repopulating adult WBM (91).

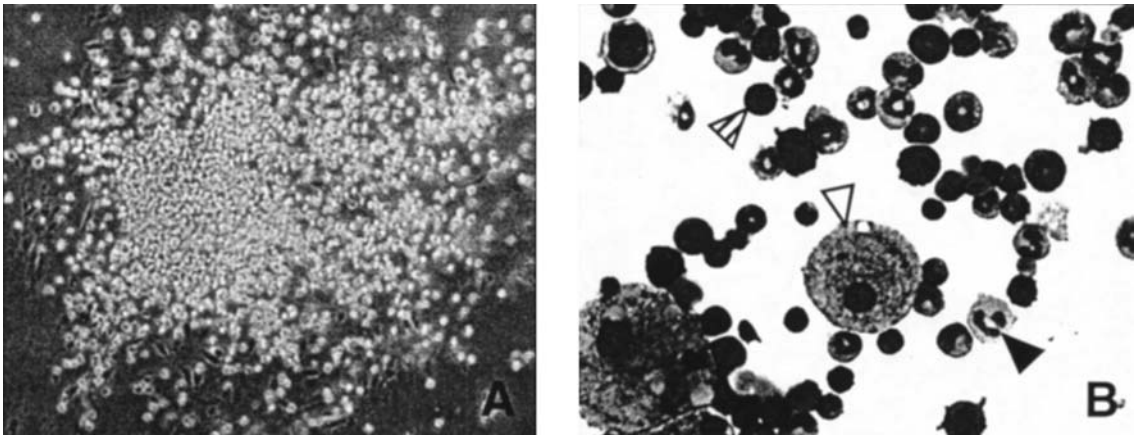

Figure 3 Representative in vitro colonies. (A) Typical appearance of a WBM-derived myeloid colony cultured for 7 days in methylcellulose supplemented with fetal calf serum and cytokines. (B) CFU types can be characterized by Wright-Geimsa stain, which allow the visualization of cytoplasmic and nuclear morphology. In this representative experiment, a mixed CFU comprising macrophages (white arrow), differentiated granulocytes (black arrow), and progenitors (striped arrows) is visualized.

To quantitatively evaluate in vivo HSC activity, donor and recipient PB reconstitution must be distinguishable because lethal irradiation does not ablate 100% of endogenous WBM. The CD45 system of congenic mice can simultaneously distinguish between donor and recipient and verify the PB identity of analyzed cells (92). CD45 is a cell surface tyrosine phosphatase exclusively expressed by nucleated hematopoietic cells (93). Monoclonal antibodies can discriminate between multiple alleles of CD45. Typically, CD45.1 WBM cells are transplanted into irradiated CD45.2 mice, and flow cytometry is used to examine recipient PB for CD45.1 positive cells. Repopulation of the different compartments of the PB can be analyzed by costaining with PB lineage markers. This model can be made semiquantitative by injecting known numbers of both a ''test'' population of CD45.1-derived cells with ''competitor'' CD45.2-derived WBM into CD45.2 recipients. The HSC activity of the test population and the competitor WBM is then compared (94).

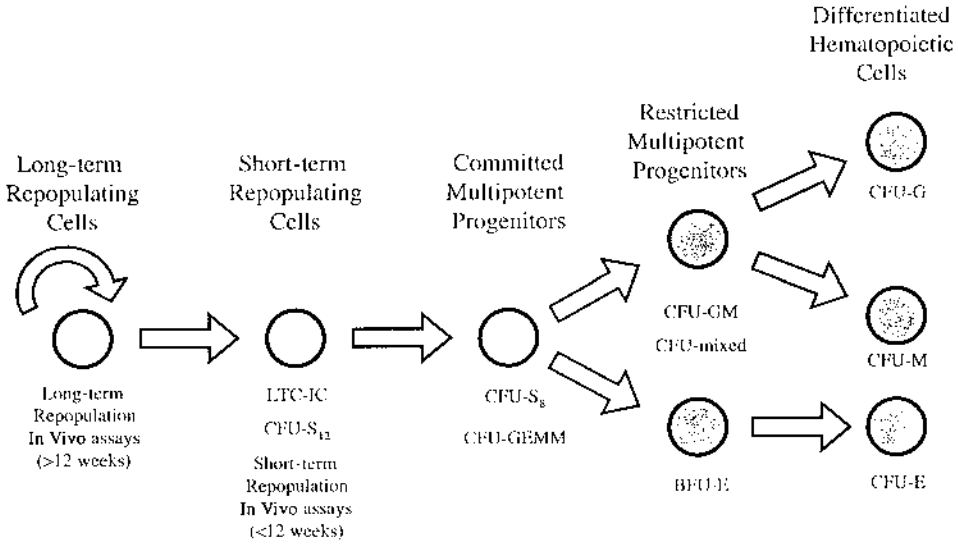

Figure 4 Summary of in vivo and in vitro primitive hematopoietic activity assays. Depicts which primitive hematopoietic stem cell and progenitor populations are assayed by various in vitro and in vivo assays.

Murine models have also been developed to examine the primitive hematopoietic potential of human WBM. In the SCID-hu model, pieces of either human fetal thymus or human fetal lymph nodes are implanted under the kidney capsule of SCID mice (95). SCID mice do not produce graft-rejecting lymphocytes and are therefore amenable to transplantation with non–self-tissues or cells. This model allowed the in vivo differentiation of human B and T cells to occur transiently (95).

Bg/nu/xid mice also do not produce graft-rejecting lymphocytes or natural killer (NK) cells (96). Transplantation of human WBM cells into sublethally irradiated bg/nu/xid mice yielded human lymphoid engraftment for 5 weeks. SCID mice injected with human growth factors and nonobese diabetes (NOD)/SCID mice (SCID mice crossed with nonobese diabetic mice that lack NK cells) can maintain higher and sustained levels of engraftment with human lymphoid cells (97,98). Also, mice transplanted with SCID WBM can support grafts of human lymphoid cells (99). Most human in vitro assays only detect myeloid differentiation because the architecture of the bone marrow and thymus, which are difficult to recreate in vitro, are required for B and T cell development. Thus, murine models of human hematopoietic differentiation are key to studying human lymphoid development in the laboratory.

Fetal sheep have also been used to assess human HSC activity in vivo. The immature immune system of the fetal sheep is tolerant to human cells transplanted in utero. Human fetal liver-derived HSC have been found to stably engraft the bone marrow and PB of primary and secondary fetal sheep recipients for >2 years posttransplant (100,101).

IV. FUNCTION OF HSC

The HSC compartment is functionally heterogeneous and varies by repopulation kinetics and persistence. In this section, we discuss the functional heterogeneity of HSC.

A. HSC Heterogeneity

Some cells capable of long-term bone marrow repopulation are unable to rescue mice from lethal irradiation (102). The ''2 waves of hematopoiesis'' theory explains this observation: bone marrow reconstitution consists of early, short-term reconstitution that rescues from irradiation and late, long-term reconstitution, which sustains the PB for an organism's lifetime (102). Long- and short-term reconstituting HSCs are phenotypically distinct: long-term HSCs are $Rhod^{Low}Mac\text{-}1^{Neg}CD4^{Neg}$, whereas short-term HSCs are $Rhod^{Med}Mac\text{-}1^{Low}CD4^{Low}$ (35,103).

Retroviruses have been used to examine the kinetics of bone marrow repopulation by HSCs. Every retrovirally infected cell is distinguishable by the unique genomic integration site of the retrovirus. The clonal nature of bone marrow-derived CFU-C in W/W mice transplanted with retrovirally transduced WBM was initially observed this way (104). Treatment of WBM with 5-FU made HSCs more prone to retroviral transduction via induction of the cell cycle (105), making it possible to observe clonal reconstitution of multiple hematopoietic tissues and verify the primitive, multipotent nature of the repopulating cell (105). Hematopoietic repopulation of PB fluctuates over time: some clones persist long term, whereas others contribute only to initial reconstitution before waning (106,107) (Fig. 5). Fluctuation lasts 4 to 6 months posttransplant and equilibrates with a few dominant HSC clones stably reconstituting the PB out to 16 months posttransplant (28). Thus, hematopoietic reconstitution is a dynamic process.

B. Self-renewal

To maintain lifelong hematopoiesis, the HSC compartment must be replenished. Self-renewal of primitive hematopoietic cells was first demonstrated when spleen colonies were shown to generate additional spleen colonies in vivo (80). Both human and murine CFU-C can be serially replated and form additional CFU-C (53,108), and bone marrow can be serially transplanted into secondary recipients without a notable loss in CFU-C potential (109). Also, a single long-term repopulating

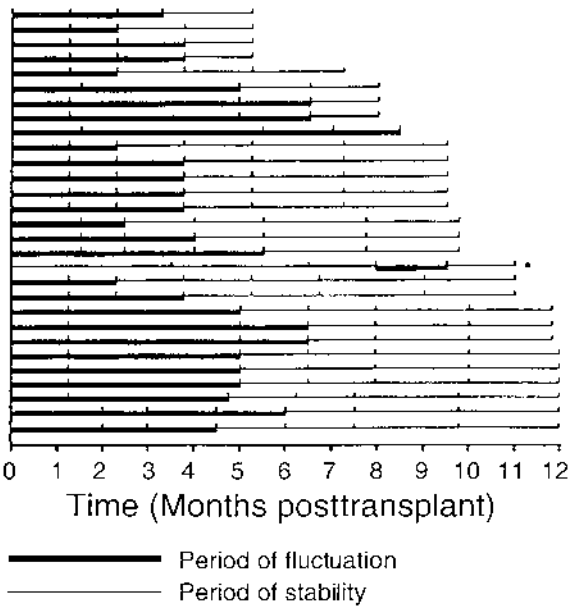

Figure 5 Clonal retroviral analysis of temporal HSC repopulation dynamics. In this experiment, retrovirally transduced HSC were transplanted into lethally irradiated mice. The contribution of clones of HSCs to peripheral blood was followed for 1 year. The thick bars represent the period posttransplant during which many HSC clones were intermittently contributing to the peripheral blood. The thin bars represent the period post transplant where a few dominant HSC clones took over the repopulation of the peripheral blood. (From Ref. 226.)

HSC can reconstitute both primary and secondary recipient mice (110).

However, the potential for self-renewal is not unlimited: serially injected CFU-S progressively lose their ability to form additional CFU-S (80). Murine bone marrow is exhausted of repopulating potential after 4 to 5 consecutive transplants (111,112), and bone marrow-derived from animals that have recovered from treatment with busulfan (an alkylating agent that ablates WBM) loses its ability to serially repopulate (113). A 7 to 8 fold loss in HSC repopulating potential after only 2 serial transplants has also been reported (114). The reduction in repopulating activity has been attributed to an exhaustion of the inherent self-renewing potential of HSCs. However, self-renewal may also be regulated by the bone marrow environment: the greatest reduction in the capacity to self-renew after serial transplantation was observed when small numbers of HSC were transplanted (115). This implies that a regulatory feedback loop may suppress self-renewal in favor of differentiation when demand is high. Also, long-term repopulating HSCs can expand 8400-fold, indicating a huge inherent potential for self-renewal. Thus, external factors must play a role in reducing HSC repopulating potential after serial transplantation (116).

C. HSCs and Aging

HSCs from old and young mice have equal repopulating potential in competitive bone marrow transplants (111). However, the HSC compartment appears, phenotypically, to increase in size in older mice, perhaps because older HSCs are more frequently in cycle than younger HSCs (117,118). Also, fetal liver-derived HSCs are reportedly 5-fold better at long-term reconstitution than bone marrow-derived HSCs (119). Thus, although in adults, old and young HSCs do not differ in activity, embryonic and adult HSCs may be functionally distinct.

HSCs from distinct strains of mice differ in their repopulating potential: C57Bl/6 HSCs outcompete DBA HSCs (120). Also, C57Bl/6-derived HSCs override DBA-derived HSCs in hematopoietic repopulation as the mice age (120). C57Bl/6 are a ''long-lived'' strain of mice relative to DBA mice, and DBA stem cells divide faster than C57Bl/6 stem cells (121). Based on these data, DBA-derived HSCs are considered to age more rapidly than C57Bl/6-derived HSCs (122). Thus, individuals with active HSCs may age more rapidly than individuals with largely quiescent HSCs. Genes regulating the cell cycle of stem cells may also regulate aging.

D. Cell-cycle Status

Most HSCs are quiescent. This is demonstrable by HSC resistance to 5-FU treatment (123–125). 5-FU or hydroxyurea (which kills all S-phase cells) treatment enriches both the engraftment potential of WBM and the frequency of bone marrow-derived CFU-S and CFU-GM (26,124–126). Ninety-nine per cent of human $CD34^{Pos}$ PB HSC are in G_0 (127) and, when mobilized human $CD34^{Pos}$ PB HSC are fractionated into G_0 and G_1 compartments, only the quiescent G_0 compartment engrafts NOD/SCID mice (128). Some studies report cell-cycle induction of HSCs diminishes their ability to repopulate WBM and form CFU-S (129–131), whereas other studies show that HSCs in both G_1-G_0 and S-G_2M have equivalent abilities to repopulate WBM (36). Follow-up reports show that, although both G_1-G_0 and S-G_2M HSCs are capable of short-term hematopoietic reconstitution, only G_1-G_0 have long-term engraftment potential (132). Thus, long-term repopulating HSCs are generally considered mitotically quiescent.

E. Homing of HSC to WBM

Injected HSCs must home to a specific niche to repopulate ablated bone marrow. Homing of HSCs is not well understood, but several factors have been implicated in this process. Selectins and integrins, adhesion molecules, and their receptors, respectively, participate in bone marrow homing of HSCs in primates and mice (133,134). Also, human HSC engraftment of NOD/SCID mice depends on the expression of CXCR4, a chemokine receptor (135). Stem cell factor is expressed in the bone marrow microenvironment, suggesting that c-kit may also regulate HSC trafficking (136).

F. HSC Genomics

Recently, global gene expression in HSCs has been examined. Gene expression in fetal liver HSCs has been examined by construction of a subtracted cDNA library (137). The HSC transcripts were analyzed for sequence homology to existing databases and an online web-accessible ''stem cell'' database published (137). Genes common to different populations of stem cells (e.g., neural stem cells, embryonic stem cells) have also been identified using subtractive hybridization (138) or Affymetrix gene chips (139,140). About 260 genes commonly expressed by HSCs, neural stem cells, and embryonic stem cells, were identified, although only about 10% of the genes reported were found by both groups. Future work must focus on identifying stem cell-specific genes crucial to stem cell identity.

V. PLASTICITY IN DIFFERENTIATION OF HSCs

Traditionally, stem cells are viewed as being restricted to differentiate into the cells of their tissue from which they were derived. However, some adult stem cells may be able to differentiate into a variety of tissue types. For example, WBM was found to contribute to the regeneration of muscle fibers in mdx mice, a murine model of muscular dystrophy (141). The HSC was later identified as the bone marrow-derived cell responsible for this myogenic activity (75). Although HSC myogenic potential is now considered extremely limited (142), these initial studies heralded a new era of research on adult stem cell ''plasticity'' in differentiation.

HSCs have since been purported to differentiate into liver, epithelium, endothelium, and cardiac muscle (143–150) (Fig. 6). However, there is some controversy regarding the prevalence as well as the existence of this phenomenon (151–154). It is likely that HSC are capable of a limited degree of trans-differentiation under specific conditions, but this must be borne out by further research.

VI. DISEASES WITH ABNORMAL HSC FUNCTION

Because HSCs give rise to all cells of the blood system, a genetic defect in HSCs will affect all the progeny cells. If the defect can be corrected in the HSC, it follows that all the progeny cells will be normal. Because HSCs can be relatively easily isolated, transduced, and reinfused, they have been believed to be an ideal candidate for gene therapy. There are a number of mouse models for human immunodeficiencies that have served as templates for gene therapy trials in humans (Table 1), and these are discussed below.

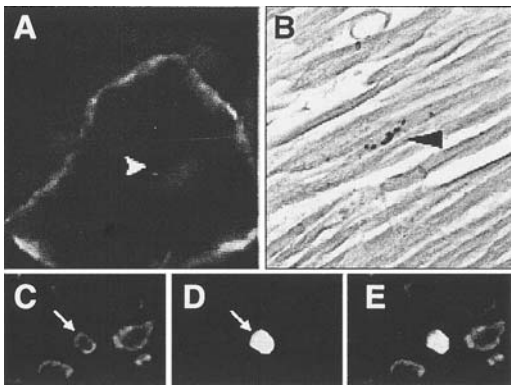

Figure 6 Examples of adult stem cell trans-differentiation. The contribution of adult stem cells to disparate tissues is typically visualized 3 ways: by transplanting male cells into female tissues and using Y-chromosome specific in situ hybridization to distinguish donor cells, by transplanting β-galactosidase positive cells and looking for the incorporation of ''blue'' cells, or by transplanting cells that express green fluorescent protein (GFP) and looking for fluorescent cells. Panel A shows the contribution of a male HSC to female dystrophic skeletal muscle (75). The red dot (white arrow) is the Y chromosome and the green staining is dystrophin, indicating that the incorporation of the HSC restored normal dystrophin expression, which is absent in these mice. Panel B shows the contribution of beta-galactosidase positive HSC to damaged cardiac muscle (145). (C–E). The white arrow in panel C is pointing out a nestin positive neuronal cell (227). Panel D reveals that this cell is derived from GFP-expressing HSC. Panel E is showing that the GFP expression and nestin staining overlap.

JAK-3 is a protein tyrosine kinase that is involved in cytokine signaling (155). It associates with the common gamma chain, which is used by the cytokines IL-2,4,7,9, and -15 (155). Lack of JAK-3 function results in low T and NK cell numbers, leading to opportunistic infections and often death (156). It is estimated that about 7% of human SCID cases are due to defective JAK-3 expression. JAK-3 null mice have a similar phenotype to humans, although they have low levels of B cells as well (157–159). One group has used retrovirus expressing JAK-3 to transduce murine HSC, which are then transplanted into JAK-3 null mice. These studies have shown that restoration of JAK-3 by this method increases the numbers of B and T cells. Most important, these mice were able to generate normal immune responses after immunization (160) and after challenge with influenza A virus (161). Because human JAK-3 deficient patients are often prone to virus infections, these studies show that the use of gene therapy of HSCs to restore JAK-3 expression may be applicable in humans.

Another immunodeficiency in which gene therapy may be useful is Wiskott-Aldrich syndrome (WAS). WAS is an X-linked disorder that is characterized by thrombocytopenia, eczema, and abnormalities in cell-mediated and antibody immune responses (162). The protein involved in this disorder is the WAS protein (WASP). Studies have shown that retroviral-mediated restoration of WASP in B cell lines, T cell lines, and primary T cells from WAS patients can partially restore normal function in these cells (163,164). Future experiments should be to transduce HSCs with the WASP protein and transplant them into WAS recipients; a murine model is available for this type of experiment.

Chronic granulomatous disease (CGD) results from a mutation in 1 of 4 proteins that make up nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase generates large amounts of superoxide in the respiratory burst. CGD patients have diminished NADPH activity; as a result, they are susceptible to bacterial and fungal infections (165). Several investigators have used mouse models for CGD to test restoration of NADPH oxidase activity by retroviral transfer of the defective protein subunit (166–169). In these studies, whole bone marrow (or bone marrow partially enriched for HSC) was transduced with a retroviral vector carrying a functional NADPH oxidase subunit gene. The cells were then transplanted into irradiated recipients that lack that gene. In all cases, NADPH oxidase activity was only partially restored, as protein levels of the expressed gene were detected at low amounts. However, this partial restoration of NADPH activity was sufficient to achieve marked improvement in defense against pathogens that would decimate mice lacking the gene entirely. One study (170) showed that retroviral transduction of a phox gene into human $CD34^+$ cells from CGD patients results in restoration of about one-half of the phox activity in these cells. Protein levels of the transduced gene were similar to those in wild-type cells.

Fanconi anemia (FA) is a disorder in which cells are hypersensitive to DNA cross-linking agents such as mitomycin C (171). The disease, which is due to mutations in the FANC-

Table 1 Animal and In Vitro Models of Gene Therapy of HSC Disorders

Deficiency	Vector	Outcome	References
JAK-3 mutations	Retrovirus	Complete correction in vivo in mouse	(160,161)
WAS	Retrovirus	Partial in vitro correction of human cells	(163,164)
CGD	Retrovirus	Partial correction in vivo in mouse, partial correction in vitro of human cells	(166–170)
Fanconi anemia	Retrovirus, AAV (in vitro)	Complete correction in vivo in mouse, complete correction in vitro with human cells	(172,173)
Sickle-cell anemia	Lentivirus	Complete correction in vivo in mouse	(174)
Beta-thalassemia	Lentivirus	Complete correction in vivo in mouse	(175)

C gene, often results in bone marrow failure and predisposition to malignancies (171). A retrovirus carrying a functional FANC-C gene was used to transduce bone marrow from FANC-C −/− mice (172). The cells were then transplanted into FANC-C −/− mice and subjected to treatment with mitomycin C. Mice receiving the corrected gene exhibited normal blood counts, whereas mice not receiving the gene exhibited decreased blood counts, leading to death. Therefore, phenotypic correction was established by retroviral transfer of the FANC-C gene. In addition, proof-of-principle studies using human $CD34^+$ cells from FA patients have demonstrated that adeno-associated virus (AAV) (173) or retroviral (173) delivery of the human FANCC gene can restore resistance to mitomycin C in vitro. Similarly, sickle-cell anemia and β-thalassemia have been corrected in mouse models by lentiviral transduction of HSCs (174,175).

In summary, gene therapy of HSCs in mouse models of human diseases has provided proof-of-principle evidence that gene therapy can be used to correct some genetic disorders, although the transition from mouse to human studies can be quite difficult. The use of mouse models for human disease does provide a template to design and test gene therapy protocols for future human trials.

A. Potential Settings to Take Advantage of Plasticity for Cell Therapies

Recent studies purporting that HSCs can differentiate to nonhematopoietic cell types have suggested that HSCs may be used as a cell-based therapy to treat disorders pertaining to nonhematopoietic cells (Table 2). The most definitive demonstration of plasticity to date is from transplantation of highly enriched HSCs into mice lacking the enzyme FAH (143). These mice are a model for hereditary tyrosinemia type I, a liver disorder in humans. Transplantation of wild-type HSCs into FAH −/− mice could reverse the lethal phenotype in some mice. The HSC compartment in bone marrow was the only fraction that had this effect. If extrapolated to humans and shown to occur at a reasonable efficiency, it is conceivable that HSCs or bone marrow transplantation could be used to treat nonhematopoietic diseases. However, as discussed above, extensive research must first be performed to confirm the phenomenon and improve the efficiency.

VII. GENE TRANSDUCTION OF HSC

HSCs give rise to all cell types of the blood and can be isolated, transduced, and reinfused back into the donor. Therefore, the correction of a genetic defect in HSC will be passed along to all its progeny, making the HSC a prime target for gene therapy. Many different viral vectors are available for use in HSCs. The selection of a proper viral vector is made based on the type of study or therapy being performed. Each viral vector has different properties, advantages, and disadvantages for use in gene therapy; these are reviewed below and in Table 3.

A. Retrovirus and Lentivirus

Stable expression of HSC can be achieved by retroviral and lentiviral transfer, which are reviewed in more detail elsewhere in this book. A main advantage of a retroviral vector is that it integrates into the chromosome of the transduced cell, resulting in stable expression. This is important for long-term expression because a nonstable vector will be lost when the HSC begins to proliferate upon activation. A main disadvantage is that the HSC must be cycling in order to be transduced with a retrovirus, and most HSCs are quiescent. Enforced cycling of HSCs leads to increased differentiation, so many HSCs are lost during retroviral transduction. These problems can be overcome by using a lentiviral vector, which can transduce nondividing cells.

Other advantages of retroviruses and lentiviruses include the ability to change the envelope of the viruses, allowing for infection of a wide variety of cell types. A disadvantage of these viruses is that stable expression may not be desired. For example, some proteins involved in differentiation need to be expressed and subsequently down-regulated to cause differentiation.

B. AAV

AAV can integrate into the genome or persist as an episome, establishing stable expression of a gene of interest, and it can

Table 2 Use of HSC for Treatment of Disorders in Nonhematopoietic Tissues

Disease	Treatment	Outcome	References
Cardiac infarct	Partially enriched HSC injected into heart or treatment with mobilizing cytokines	Partial correction of infarct	(146)
Hereditary tyrosinemia type I	Transplantation of HSC	Correction of lethal phenotype in some recipients	(143)
Acute or chronic muscle injury	Transplantation or direct injection of HSC or whole bone marrow	Few donor-derived muscle cells	(75,141,224)

infect dividing or nondividing cells. An additional benefit of AAV is that it is not toxic to cells. Disadvantages of AAV include difficulty in making high-titer, large-scale stocks free from contamination with wild-type virus (176).

AAV has been used by many groups to infect both mouse and human HSC. In mouse, AAV is believed to stably transduce HSCs (177–180). However, detailed studies looking at the efficiency of transduction of mouse HSCs have not been performed. AAV has been used far more often for transduction of human HSCs. Reports of transduction efficiency of human HSCs by AAV has ranged from 4% to 80% (181–184). The discrepancies in these studies can be partially attributed to differences in source of HSCs (bone marrow, umbilical CB, peripheral blood of mobilized patients), MOI, virus preparation, time of incubation, and the use of cytokines. The use of an AAV vector to correct a defect in human HSCs has been reported (173). This study showed in vitro correction of a phenotype of FA in $CD34^+$ cells isolated from an FA patient. It is possible that use of AAV in vivo in humans will increase as improvements in AAV production matures (176).

C. Adenovirus

Adenovirus (Ad) can transduce a wide variety of either dividing or nondividing cells. Ad can carry about 7.5 kb of transgene insert, although ''gutless'' vectors lacking Ad genes can carry over 30 kb. Other benefits of Ad include high expression levels and the ability to make large quantities of high-titer virus. Disadvantages of Ad include toxicity to transduced cells, although toxicity can be decreased by using gutless vectors. Ad expresses genes only transiently because they do not integrate into the genome. This makes adenovirus unsuitable for cases in which long-term, stable transgene expression is required.

Adenovirus has been used to transduce human HSCs. As early as 1985 (185), experimenters determined that hematopoietic cell lines could be transduced by adenovirus, and primary HSCs were shown to be transduced by Ad vectors (186). Subsequent experiments showed that Ad serotype 5 (Ad5) vectors could transduce human $CD34^+$ cells at moderate efficiencies, ranging from 5% to 45% (187–191). These studies also reported little toxicity in in vitro colony-forming ability, except at high MOI. The studies varied in MOI used, culture time, and source of HSCs. The use of cytokines during culture time to increase transduction efficiencies has yielded conflicting reports (190,192). Transduction of the more primitive $CD34^+$ $CD38^-$ cells showed transduction rates ranging from 15% to 45% (188,191). Studies using polycationic lipids to coat Ad [virus–polycation complex (VPC)] reported higher transduction efficiencies compared with virus alone (193–195). Due to the difficulties in testing human HSCs in vivo, most of these studies tested the toxicity of the virus by culturing transduced or nontransduced cells in vitro. However, one study transplanted transduced HSC into NOD/SCID animals and showed multilineage engraftment similar to nontransduced cells (195).

Most of the earlier studies used Ad5 to transduce human HSCs. However, many other serotypes exist. Thus, tests with

Table 3 Properties of Viral Vectors for Transducing HSC

	Retrovirus	Lentivirus	AAV	Adenovirus
Advantages	Stable expression	Stable expression, can infect dividing and nondividing cells	Stable expression, nontoxic, infects nondividing cells	Large insert capacity, high expression levels, high titers
Disadvantages	Infects only dividing cells	Difficulties in large-scale production, safety concerns, low expression levels	Low titers, low transduction efficiency	Transient expression, toxicity

chimeric vectors, that is, vectors constructed with parts of different serotypes, were studied. The Ad5F35 vector, consisting of the Ad5 vector with an Ad35 fiber protein, displayed highly efficient transduction of human HSCs compared with Ad5 vectors (196,197). The Ad5F50 vector also displayed greatly increased transduction efficiencies (198), up to about 90%. Ad5F11 vectors transduced about 70% of $CD34^+$ cells (199).

In contrast to the work on human HSCs, little has been reported on Ad transduction of mouse HSC heretofore. One group has shown that about 38% of a partially purified mouse HSC population can be transduced by Ad5 (200). Our group has detected about 32% transduction of highly purified mouse HSC population by Ad5. Transduced cells have normal in vitro activity. In vivo, transduced HSCs show multilineage contribution to recipients, but at a level of about half that of nontransduced cells (201).

Some groups have advocated the use of Ad to purge cancer cells from bone marrow. Such a technique would be useful for cancer patients undergoing autologous HSC transplantation to eliminate cancer cells from the autograft. Studies have shown that Ad5 infects cancer cells much more efficiently than HSCs, and can be used to selectively purge cancer cells from a bone marrow/cancer cell mixture without harming HSC activity (202–205). These reports appear to conflict with the reports cited above (206). It is probable that different experimental settings can be used to achieve either transduction of HSC or purging of cancer cells in the bone marrow environment (207). Another possibility is that Ad5 targets cancer cells more efficiently than HSC and therefore infects cancer cells at the expense of HSC in a mixed culture.

Experiments have been done on targeting the adenovirus to specific cell types in a mixture. For example, c-Kit, a cell surface receptor expressed on HSCs, is not expressed on most cell types in the bone marrow. By biotinylating Ad and SCF (the ligand for c-kit) and using avidin to attach SCF to Ad, transduction of hematopoietic cell lines was increased (208). In addition, biotinylated antibodies to c-kit could also be attached to Ad to increase transduction efficiency (208). Similarly, another study showed that by adding avidin to the Ad, and biotinylating SCF, an Ad-SCF vector could be created (209). This vector binds c-kit+ cells more efficiently, leading to more efficient transduction of human $CD34^+$ cells (209). Another group has circumvented the problems associated with biotinylating Ad chemically by developing a system in which Ad is biotinylated metabolically as it is being produced. A biotinylated anti CD71 or CD59 antibody was linked to this vector and used to greatly increase the transduction of a hematopoietic cell line (Parrot and Barry, Molecular Therapy, in press).

Hybrid vectors, which combine the favorable qualities of 2 vectors into 1 vector, are discussed in detail elsewhere in this book. For the purpose of this chapter, it is suffice to say that recent work illustrating the use of Ad for transduction of HSCs leads the way for Ad-based hybrid vectors that may allow for stable transduction of HSCs. These systems are based on an Ad vector carrying sequences from other viruses (AAV, retrovirus, or EBV) that cause stable expression of the gene of interest (210–214).

VIII. CURRENT STATUS OF GENE THERAPY FOR HUMAN HEMATOPOIETIC DISORDERS

There are many human hematopoietic disorders that could potentially be treated using gene therapy (Table 4). In most of these diseases, transplantation of bone marrow from a healthy donor could cure the patient. However, a suitable donor is not always available, and the transplantation procedure itself can be toxic. Therefore, the use of gene therapy as an alternative treatment would be a significant advance. The first disorder in which a gene therapy protocol was used in clinical trials was adenosine deaminase (ADA)-SCID, which occurs due to deficiency in ADA expression, resulting in SCID. Patients can be successfully treated by receiving exogenous ADA (PEG-ADA), but must continue this regimen for life. Early gene therapy trials used a retrovirus expressing an ADA minigene to transduce umbilical CB or bone marrow cells and/or peripheral blood lymphocytes (215–218). Although some transduced cells persisted and were functional, patients were still dependent on PEG-ADA treatment; removal of PEG-ADA resulted in a relapse into immunodeficiency.

Correction of ADA-SCID by gene therapy has now been improved with better HSC transduction and transplantation protocols (219). Two patients were reinfused with their transduced bone marrow after a nonmyeloablative conditioning regimen. Normalization of the patients' immune system in the absence of PEG-ADA was achieved and had been sustained for about 1 year at the time of the report (219).

The first successful human gene therapy trials were performed on patients with X-linked SCID (SCID-X1). SCID-X1 is caused by mutations in the common gamma chain, which is a signaling chain used by several cytokines. Lack of this chain results in drastically reduced T and NK cell development, leading to SCID. $CD34^+$ cells from SCID-X1 patients were transduced with a retrovirus carrying the common gamma chain and infused, without myeloablation, into the patients. Nearly 1 year after this, immune function was normal and

Table 4 Clinical Trials Using Gene Therapy of HSC

Disorder	Vector used	Result	References
ADA-SCID	Retrovirus	Partial correction	(216–218) (215)
SCID-X1	Retrovirus	Complete correction	(220,221)
CGD	Retrovirus	Very few cells corrected	(225)
FA	Retrovirus	Partial transient correction	(223)

patients were living normal lives at home (220,221). However, 1 of the 10 patients treated in this manner has developed a leukemia-like disease that is possibly due to integration of the retrovirus into the host chromosome (222), raising questions as to the safety of integrating viruses for gene therapy.

Retroviral transduction of HSCs has also been conducted for disorders such as CGD and FA (223). Although these studies have shown long-term expression of the defective genes, the expression level and transduction rates have been too low to show any lasting clinical benefit.

Although the successful trials with SCID-X1 and ADA-SCID patients reflect the promise of gene therapy, failures of other trials represents the obstacles needed to be overcome. One of these obstacles is the frequent problem of low expression levels of the transgene following transduction. However, as demonstrated by the mouse CGD experiments described earlier, low transgene expression may be sufficient to restore function to the affected cells. In other situations, even low levels of transgene expression may be sufficient for preferential selection of the transduced cells, bypassing the need for high transduction efficiencies and high gene expression levels. Another question is how many HSCs must be transduced in order for lasting therapeutic effect. It is likely that these questions must be empirically answered for each disorder being treated.

The possible retroviral-linked leukemia in one patient outlines another problem in gene therapy that needs to be addressed (222). Studies regarding the safety of different integrating retroviruses and the study of stable expression via nonintegrating episomes may provide more information on the plausibility of safe gene therapy treatments. Although gene therapy of HSCs has shown great promise, these questions must be answered before this type of treatment can be used on a regular basis.

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44

Gene Marking Studies of Hematopoietic Cells

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I. INTRODUCTION

The transfer of genetic material into a target cell offers a potentially powerful strategy in the treatment of a wide variety of diseases. Although gene marking does not confer therapeutic benefit, much of the biological information gained from these trials is proving to be valuable for improving the outcome of therapies such as stem cell transplantation (1,2). Gene marking can be used to track the in vivo behavior of almost any tissue (3), and the success of the strategy depends on tracking the marked cell not only for its entire lifespan, but also for the lifespan of all its progeny. The vectors used must therefore integrate into the host cell DNA and efficiently replicate with the cell. For this reason, the majority of marker studies performed to date have used murine retroviral vectors. Although these agents are able to integrate stably, they have many limitations. In particular, they are only able to integrate into dividing cells and have low transduction efficiency.

The aims of this chapter are 3-fold: (1) to identify the reasons why gene marking studies have been performed and why a continuation of this research is important to the ever-broadening field of gene transfer; (2) to discuss the vectors and marker genes employed, and the factors, which influence gene transfer efficiency and gene expression; and (3) to review the lingering concerns about the safety issues surrounding gene transfer.

II. GENE MARKING OF HEMATOPOIETIC PROGENITOR CELLS

The principle of gene marking is the transfer of a unique DNA sequence (e.g., a nonhuman gene) into a host cell (e.g., T cell, hematopoietic stem cell, etc.) allowing the gene or the gene product to be easily detected, thereby serving as a marker for these labeled cells. Detection of the marker gene may be phenotypic or genotypic. For example, the frequently used bacterial neomycin resistance (*neo*) marker gene encodes the enzyme neomycin phosphotransferase, which can inactivate neomycin or its analogs, such as G418. When cells are expressing the *neo* gene, they become resistant to G418 in culture medium and can be detected by their ability to grow in G418. The transgene can also be readily detected in these cells using the polymerase chain reaction (PCR) technique (2).

A. Gene Marking in Autologous Stem Cell Transplantation

In the first gene marking trials involving hematopoietic stem cells (HSCs), the question posed was whether relapse following autologous stem cell transplant was due to a contribution from contaminating malignant cells in the stem cell harvest (4). To answer this, a portion of the HSC product was marked at the time of harvest with a murine retroviral vector encoding the neomycin resistance gene. If the patient subsequently relapsed, it was possible to detect whether the marker gene was present in the malignant cells. Since 1991, studies have been initiated using this approach in a variety of malignancies treated by autologous HSC transplantation (4–12). As shown in Table 1, the underlying malignancies treated with marked autologous HSCs include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), neuroblastoma, lymphoma, multiple myeloma, and breast cancer.

The initial studies evaluating this approach took place at St. Jude Children's Research Hospital (SJCRH) in pediatric patients receiving autologous bone marrow transplantation (BMT) as partial therapy for AML or neuroblastoma (4,6). In both studies, one-third of the marrow was marked in a 6-h transduction protocol in the absence of growth factors. Four

Table 1 Results of Clinical Gene Marking Studies Using Hemopoietic Progenitors as the Target Cell

Study site	Target cell	Disease	Results	References
SJCRH	Marrow mononuclear cells	Pediatric acute myeloid leukemia	Presence of marked tumor cells at relapse Persistence of marked normal cells up to 5 years	13,25
SJCRH	Marrow mononuclear cells	Neuroblastoma	Presence of marked tumor cells at relapse Persistence of marked normal cells up to 5 years	15
MD Anderson Cancer Center	Marrow mononuclear cells	CML	Presence of marked tumor cells at relapse Persistence of marked normal cells up to 1 year	16
NIH	CD34-selected blood and marrow cells	Breast cancer	Presence of marked normal cells derived from both blood and marrow	18
NIH	CD34-selected blood and marrow cells	Multiple myeloma	Presence of marked normal cells derived from both blood and marrow	18
University of Indiana	Marrow mononuclear cells	AML	No marked tumor cells at relapse No significant detection of marker gene in normal cells	17
University of Toronto	Long-term bone marrow cultures	Multiple myeloma	Persistence of marked normal cells up to 2 years	31
Karolinska Institute	CD 34-selected cells	Multiple myeloma	Low-level detection only in normal cells	12
MD Anderson Cancer Center	CD34-selected blood and marrow cells	Non-Hodgkin's lymphoma	Presence of marked cells up to 9 months No marked tumor cells in 1 patient who relapsed	80

of 12 AML patients who received marked marrow relapsed. In 3 of the 4 patients, detection of both the transferred marker and a tumor-specific marker in the same cells at relapse provided unequivocal evidence that the residual malignant cells in the marrow were a source of leukemic recurrence (13). For example, in one patient the malignant blasts had the unique phenotypic features of CD34 and CD56 coexpression and a complex t(1:8:21) translocation resulting in an AML1/ETO fusion transcript that could be identified by PCR. Malignant cells sorted by this phenotype contained both the AML1/ETO fusion transcript and the transferred neomycin gene (13). In the neuroblastoma study, (6) 5 of the 9 patients relapsed, and gene marked neuroblastoma cells, coexpressing the neuroblastoma-specific antigen GD2 together with the transferred marker gene, were detected in 4 cases (14,15). In one of these patients, marked neuroblastoma cells were detected in an extramedullary relapse in the liver (15). Similarly, marked malignant cells coexpressing the t(9;22) and *neo* transcripts were found in 2 patients with CML at relapse (16). These data show that in these 3 malignancies, marrow harvested in apparent clinical remission may contain residual tumorigenic cells that can contribute to a subsequent relapse. In contrast, marked cells have not been found in adult patients relapsing after autografts for ALL (17). However, for logistic reasons, only around 10% of the marrow was marked in this study and gene transfer to normal cells was very low. In a study at the National Institutes of Health (NIH), autologous transplantation for breast cancer used CD34 selected HSC from blood and marrow. In the 2 patients who relapsed, the marker gene was not found (18). This may reflect a lower efficiency of the marking of breast cancer cells, or the $CD34^+$ selection procedure used may have reduced contamination with malignant cells. Alternatively, relapse in breast cancer may occur predominantly from residual disease in the patient.

Therefore, as only modest levels of gene transfer into malignant progenitor cells are obtained, a definitive conclusion about the contribution of marrow-based disease to recurrence can only be drawn if a relapse is marked. Unmarked relapses might mean that marrow does not significantly contribute to recurrence or may mean that relapse is generated by only a few marrow-derived malignant cells that have escaped being marked because of the inefficiency of the transduction process.

III. THE USE OF MULTIPLE VECTORS

A. Double Gene Marking to Monitor Purging

The overall implication of the above studies is that residual malignant cells can contribute to relapse, and the outcome of HSC rescue may be improved by strategies targeted at residual malignant cells (19). One means of achieving this is purging of the transplanted marrow and gene marking techniques are useful in the evaluation of the purging technologies. In second-generation studies, 2 genetically distinct marker genes were used to compare either marrow purging vs. no purging or 2 different purging techniques (12,20,21). Two closely related vectors, G1N and LNL6, were used. These vectors differ in their 3′ noncoding sequences so primers can be designed allowing the vectors to be discriminated by virtue of the differing fragment sizes they produce after PCR amplification. In the AML study, (20) 15 patients were enrolled between September 1993 and November 1996. At least 10^8 marrow cells/kg were frozen without manipulation as a safety backup. The remaining marrow mononuclear cells were divided in half and randomized to marking with G1Na or LNL6. The aliquots were then randomly assigned to the 2 purging techniques being evaluated. Initially, the pharmacologic ''gold standard'' hydroperoxycyclophosphamide (4HC) was compared with an immunologic purge by culture with interleukin-2 (IL-2) (22). Later into the study, CD15 antibodies (instead of IL-2) were used in conjunction with 4HC (23). At the time of transplant, both aliquots were reinfused. If the patients relapsed, the premise was that detection of either marker would reveal which of the compared purging techniques had failed.

Over the 3-year period, 15 patients were treated. In 5 patients, aliquots of marrow were purged with 4HC and IL-2; in 3 patients, the marrow was purged with 4HC and CD15. PCR studies on peripheral blood granulocytes and mononuclear cells consistently showed a stronger signal from the 4HC-purged fraction than that from the IL-2-purged fraction, regardless of which vector was used for marking each aliquot. These observations suggested that the 4HC-purged fraction was making a greater contribution to hematopoietic reconstitution than the IL-2-purged fraction, thus resulting in the substitution of CD15 antibodies for IL-2. Seven patients received marrow purged with 4HC alone. This was due to either insufficient number of cells harvested or unavailability of 1 clinical grade retroviral vector. In all, 3 of the 15 patients relapsed. Two patients relapsed early at 2 and 3 months and were noninformative, as marked malignant cells were not detected. A third patient relapsed at 20 months and his blasts were also negative (24). The *neo* gene was detected in normal hematopoietic and immune system cells at a lower level than in the studies using unpurged marrow.

IV. GENE TRANSFER TO NORMAL CELLS

These marking studies also provided information on the transfer of marker genes to normal hematopoietic cells and showed that marrow autografts contribute to long-term hematopoietic reconstitution after transplant (2,19) (Table 1). Long-term transfer up to 9 years has been seen in the mature progeny of marrow precursor cells, including peripheral blood T and B cells and neutrophils (24). It was also detected in lymphoblastoid cell lines and cytotoxic T cell lines derived from these patients. However, the level of gene transfer varied and was highest in marrow clonogenic hematopoietic progenitors, where an average of 6% of myeloid colonies were G418 resistant at 3 months. The levels of transfer into marrow progenitors are higher than predicted from animal models and may be attributed to the fact that marrow was harvested during regeneration after intensive chemotherapy, when a higher than normal proportion of stem cells are in cycle (16). In the mature peripheral blood cells, the level of gene transfer was however some 5-fold lower than in the marrow progenitors with levels of 0% to 1% seen (25).

Useful information can be gained from these initial studies, leading to the development of more complex gene marking protocols (Table 1). These include (1) double gene marking to allow simultaneous study of 2 distinctly treated cell populations (e.g., of unmanipulated HSCs vs. stem cells treated with growth-stimulatory agents); and (2) the use of more purified populations of early progenitor cells to allow the ''true'' pluripotent tem cell to be identified phenotypically and to increase the efficiency of gene transfer by more precise tailoring of transduction conditions.

A. Double Gene Marking to Compare Long-term Reconstitution from Different Populations of Hematopoietic Progenitor Cells

No in vitro assay can yet assess the capacity of a cell population to produce short- and long-term repopulation of humans. The use of double gene marking with distinguishable vectors in a single patient potentially allows (1) comparison of these properties between different sources of putative stem cells, such as peripheral blood and marrow; (2) determination of the function of stem cell subpopulations (e.g., $CD34^+CD38^+$ vs. $CD34^+CD38^-$ progenitor cells); and (3) the consequences of ex vivo manipulation, such as culture of putative stem cells on stromal support or with cytokines. By using the 2 distinguishable retroviral markers, it is possible to compare quantitatively in vivo and within each patient the short- and long-term reconstituting capacity of different populations of HSC.

One aim of clinical stem cell transplantation is to minimize the period of marrow aplasia posttransplant by increasing the number of progenitor cells infused. This approach is only of value if progenitor cell manipulation does not induce loss of self-renewal capacity when the treated cells are reinfused. One strategy is to expand the progenitor cells at least 50-fold ex vivo using stimulatory cytokines (18,26) and/or stromal (24,27) components. There are however concerns that stimulatory cytokines will not only induce primitive cells to expand, but will also cause them to differentiate and lose their capacity

for self-renewal. In other words, the cytokine stimulation ex vivo may produce a faster initial engraftment but at the cost of later graft failure. Gene marking can be used not only to address concerns about the effects of growth-stimulating agents on pluripotent progenitor cells and long-term engraftment, but also to determine whether ex vivo stimulation increases gene transfer efficiency.

The NIH study by Dunbar et al. (18) compared the reconstitution of peripheral blood and bone marrow in patients receiving autologous HSC rescue for myeloma or breast cancer. They differentially marked mobilized peripheral blood and marrow $CD34^+$ cells using 2 distinguishable *neo* containing retroviral vectors and infused both populations of stem cells into the same patient. An average of 21% of colony-forming units (CFUs) were transduced using a 3-day culture in the presence of stem cell factor (SCF), IL-3 and IL-6. The level of gene transfer into committed progenitor cells post transduction was as high as 50% using this approach. However, the engraftment of the marked cells was not accelerated compared with the earlier SJCRH studies (2,13,20,28) in which growth factors were not used. In addition, although vector sequences were found in myeloid and lymphoid cells at 0.01% to 0.001% for up to 18 months, these levels were lower than the earlier SJCRH studies (29,30). These findings may reflect the different patient population both by virtue of age (adult vs. pediatric) and disease (breast cancer/myeloma vs. leukemia/neuroblastoma). Alternatively, this may indicate that that culture with growth factors commits transduced cells to differentiation, so that high-level, long-term engraftment is not obtained.

Although the study by Dunbar et al. showed low levels of gene transfer detected in vivo, the marker gene derived from peripheral blood and marrow HSC demonstrated that each can contribute to long-term recovery (18). It is of interest that the marked peripheral blood stem cells contributed to hematopoiesis earlier and for a longer period compared with the gene marked marrow cells. It is possible that this phenomenon may be related to physiological differences in these 2 populations of HSCs. This includes the ability of each HSC to contribute to hematopoiesis, the cell-cycle status of the HSC, or the susceptibilty of the HSC to retroviral transduction (27).

Another promising method by which progenitor cells may be expanded and their transducibility increased is the initiation of long-term cultures or the addition of stromal support components to culture. One group (11,31) established marrow in long-term cultures where an autologous stroma developed over the course of 3-week culture. The cultures were transduced weekly. Initially, a mean of 16% of gene marked progenitors were seen in the early posttransplant period. This level dropped to 3% by 2 years posttransplant, and only 0.01% of the circulating blood cells were marked.

Although the use of double gene markers is in its infancy, and is hampered by the complexity of the studies, the approach remains a powerful way of investigating the biology of different stem cell populations and of determining the consequences of stem cell manipulation.

B. $CD34^+$-selected HSC Transplantation

In the context of gene marking, the justification for $CD34^+$ selection is that it reduces the required volume of vector supernatant. It may also serve as a means of marrow purging in patients in whom the malignant cells are $CD34^-$ or coexpress $CD34^+$ and 1 or more lineage (lin) commitment antigens. The marking approach can be used to discover the relative ability of distinct populations of $CD34^+$ cells to produce long-lived, multilineage reconstitution following reinfusion. The strategy can also be used to determine if lineage-positive and -negative CD34 subsets make a different contribution to short- and long-term reconstitution. The potential disadvantage of selecting $CD34^+$ cells or their subsets is that immune reconstitution may be delayed because mature lymphocytes are not transferred with the graft. This delay may then increase the risks of subsequent neoplastic change if undetected replication-competent retrovirus has contaminated the vector as demonstrated in an animal study (32).

The advantages of CD34 selection may therefore be counterbalanced, at least in part, by a decrease in the margin of safety for marker studies. Nonetheless, many of the more recently approved marker studies (as shown in Table 1) use $CD34^+$ cells as the vector target (12,18,29).

V. ROLE OF MARKER STUDIES TO VALIDATE EFFORTS TO INCREASE THE LEVEL OF GENE TRANSFER TO THE HSC

A. Role of the Cell Cycle

Attempts to induce HSCs to cycle generally use various cytokines/growth factor combinations. However, it has been demonstrated that culture of human $CD34^+CD38^-$ cells in serum-free media with IL-3, IL-6 SCF, Flt-3 ligand and thrombopoietin or megakaryocyte-derived growth factor (MDGF) for 9 days can increase both the number of HSCs cycling and the length of time they can be maintained in vitro (33–35). HSCs may also be induced to cycle in vivo as a normal physiological response to hematopoietic cytoablation and/or cytokine mobilization. In the SJCRH studies in pediatric patients, (2,13,20,28) a 6-h incubation with retroviral supernatant was employed for transduction. The success of the transduction efficiency and long-term persistence of marker gene expression in these studies compared with similar studies in adults (18,31) may however be attributed to the fact that HSCs from children have an endogenously higher proliferation index. There is also evidence that treatment of animals with a combination of granulocyte-stimulating factor (G-CSF) and SCF mobilizes marrow progenitor cells that are more primitive and more readily transduced than after treatment with G-CSF alone (36).

B. Role of Stromal Support Elements

Some groups add stromal supports such as a recombinant fragment of fibronectin to bring the retroviral vector and target

cell into close apposition, thereby increasing the efficiency of progenitor cell marking (37,38). Several studies have tried to address the extent of long-term engraftment of retrovirally transduced peripheral blood progenitor cells using recombinant fibronectin (38–41). One study (41) assessed the effects of recombinant fibronectin on marrow repopulating cells and on the efficiency of gene transfer using the multidrug resistance (MDR1) gene or *neo* gene to ascertain the fate of the treated cells. In this study, one-half of the enriched $CD34^+$ cells were transduced with MDR1 and the other half with *neo*. There was low-level gene marking of granulocytes (0.01%–1%) by PCR analysis up to 6 months posttransplant. In all 6 patients, there was a higher level of engraftment of MDR1-containing cells relative to *neo*, suggesting that MDR1 overexpression was beneficial to the engraftment potential of hematopoietic cells.

A recent study (38) reported the highest level of long-term engraftment of retrovirally transduced peripheral blood stem cells (PBSCs). The 11 enrolled patients were adults (17–51 years) undergoing tandem autologous PBSC transplants for germ cell tumors. In this study, MDR1 was used as a marker gene and in the hope that it may render the HSC resistant to oral etoposide, (42) thereby allowing for further dose-intensive therapy without the delay of prolonged cytopenia post transplant. The PBSCs were mobilized using G-CSF. The initial harvest was unmanipulated and used for the initial transplant. The second collection was $CD34^+$ selected, stimulated ex vivo with cytokines (SCF,IL-6,MDGF,G-CSF), and cultured with MDR-1 retroviral vector on plates coated with recombinant fibronectin (CH-296), then cryopreserved on day 5. The median gene transfer efficiency of all colonies immediately after transduction was 14% (4%–52%). At 12 months postinfusion of the MDR1-PBSCs, 4 of the 7 evaluable patients had detectable levels (5% to 15%) of transgene-containing colonies in their bone marrow samples. This study also showed that exposure of $CD34^+$ cells to CH-296 did not adversely affect either engraftment kinetics or long-term hematopoietic function.

These studies therefore indicate the feasibility of using MDR gene transfer as a means of enriching marrow for MDR-transduced cells. It remains unclear whether the approach will be valuable for protecting and selecting other cell types following chemotherapy.

VI. GENE MARKING STUDIES OF T CELLS

A. Gene Marking of Tumor-infiltrating T Lymphocytes

The first gene marking protocol was conducted to determine the fate of lymphoid cells that infiltrate solid tumors (43). These tumor-infiltrating lymphocytes (TILs), were obtained from tumor biopsies, expanded ex vivo and then reinfused. It was therefore important to determine if these infused TILs were tumor specific and ascertain their distribution and persistence at tumor sites after infusion. Initially, studies to assess the ability of TILs to traffic to and remain at tumor sites postinfusion used TILs that were radioactively labeled with ^{111}In (44,45). However, this approach requires a high labeling to enhance the detection level and duration, resulting in potentially toxic radiation exposure to cells from the gamma radiation emitted by the isotope. In addition, the half-life of ^{111}In is only 2.8 days, and it is therefore not suitable for monitoring TILs over extended periods of time (46).

It was apparent that a need existed for an alternative labeling technique. The genetic marking of the TIL ex vivo using a *neo* encoding retroviral vector helped to answer several questions about TILs in cancer therapy. After neo-marked TILs were infused into the patient and tumor sites biopsied, it was shown that large numbers of gene-marked TILs could be safely returned to patients, and that the marker could be transiently detected in the peripheral blood by PCR analysis using *neo*-specific primers (47,48). Analysis of peripheral blood and tumor deposits for presence of the marker gene suggested that TILs could persist for up to 2 months (43). Studies in one patient showed presence of the marker gene in biopsy tissue from a tumor deposit (49). In a French study, marked TILs were detected for up to 260 days and TILs were also detected in 4 of 8 tumor biopsies after therapy (50). A subsequent double-marking study compared the survival and tracking of peripheral blood lymphocytes (PBLs) and TILs by marking with distinguishable retroviral vectors (51). Both marked PBLs and TILs could be detected in peripheral blood for 4 months, and no selective homing of TILs to tumor sites compared with unmanipulated PBL was seen (51). None of these studies therefore provided support for the theory that TILs are capable of selective homing.

B. Gene Marking to Track Adoptively Transferred Cytotoxic T Cells After Allogeneic BMT

Several studies have shown the feasibility and apparent clinical efficacy of adoptive transfer of cytotoxic T cells (CTLs) directed at viral or tumor antigens (50–59). The transfer of a marker gene has allowed monitoring of adoptive transfer approaches to determine the survival of infused T cells. In addition, the T cells can be tracked to learn if they can home to sites of disease and if they mediate adverse effects such as graft vs. host disease (GVHD). The clinical results of gene marking approaches using T cells are summarized in Table 2.

Matched unrelated-donor or mismatched family-donor bone marrow transplantation results in a high risk of GVHD due to the greater genetic disparity between donor and recipient. An effective means of reducing this risk is to T cell-deplete the donor marrow ex vivo. The disadvantage of this approach is that recipients of the T cell-depleted bone marrow have delayed immune recovery and there is an increased incidence of viral infections. One such infection is Epstein-Barr virus lymphoproliferative disease (EBV-LPD), which is due to a proliferation of EBV-infected B cells that are highly immunogenic and that are not eliminated in the immunocompromised host. This complication occurs in 5% to 30% of patients

Table 2 Published Results of T Cell Gene Marking Studies

Study site	Target cell	Disease	Results	References
NCI	Tumor-infiltrating lymphocytes	Melanoma	Short-term detection marked cells	3
SJCRH	EBV-specific cytotoxic T lymphocytes	Post T cell-depleted allogeneic bone marrow transplant	Detection of marked cells up to 6 years Expansion in response to EBV reactivation Accumulation marked cells at disease sites	53, 55, 56
SJCRH	EBV-specific cytotoxic T lymphocytes	Hodgkin's disease	Detection of marked cells up to 6 months Accumulation marked cells at disease sites	57
Centre Leon Berard, Lyon, France	Tumor-infiltrating lymphocytes	Melanoma Renal cell cancer	Detection of marked cells up to 9 months No evidence of homing	50
University of California Los Angeles	Tumor-infiltrating lymphocytes and peripheral blood lymphocytes	Melanoma Renal cell cancer	Detection of marked cells up to 4 months No evidence of homing of TIL compared with PBL	51
Fred Hutchinson Cancer Research Center	Gag-specific CD8 clones	Patients with HIV	Eradication of infused cells containing Tk or hygromycin Trafficking of neo marked cells to HIV infected lymph nodes	52, 58
NIH	$CD4^+$ T cell from syngeneic twins	Patients with HIV	Detection of marked cells up to 4 months	59

receiving T-depleted marrows from mismatch family or unrelated donors. Donor-derived EBV-specific T cells were generated by culturing donor T cells with donor-derived EBV-infected lymphoblastoid cell lines (53,55,56,60). To determine whether these cells persisted and whether they caused adverse effects, they were marked with the *neo* gene before administration. Twenty-eight patients received *neo*-marked CTLs. Infusion of virus-specific CTLs produced a virus-specific immune response as documented by a fall in EBV DNA levels (53). The gene marking component of this study allowed the demonstration of the persistence of the *neo* gene in the peripheral blood for up to 78 months after injection of gene marked cells (56). Three patients who were treated for clinically evident EBV-LPD attained prolonged remission after CTL infusion, and in situ hybridization and semiquantitative PCR showed that the gene marked CTL had selectively accumulated at disease sites (55).

C. Gene Marking of EBV-specific CTLs for Relapsed EBV-positive Hodgkin's Disease

Marking of autologous EBV-specific polyclonal CTLs has also been used in a clinical study for patients with relapsed EBV genome-positive Hodgkin's disease (57). Seven patients with multiply relapsed Hodgkin's disease have received gene marked EBV-specific CTL in a phase I dose escalation study. Gene marked CTLs were found in peripheral blood up to 9 months following infusion. One patient had erosion of tumor through the L upper lobe bronchus and died 2 months after CTL infusion. In situ PCR revealed gene marked CTLs within part of the mediastinal tumor but not at the site of L upper lobe bronchus erosion. In another patient, EBV-specific CTLs were localized to a malignant pleural effusion 3 weeks after CTL infusion as assayed by gene marking. The conclusions from this study were that marked CTLs expanded in vivo and were found to persist for up to 9 months as tracked by RT-PCR analysis (61).

D. Gene Marking of T Cells in HIV Therapy

To study the survival of normal T cells in patients with HIV, peripheral blood lymphocytes from unaffected syngeneic twins were transduced with a *neo* vector and transferred to their HIV-infected sibling (59). Marked $CD4^+$ T cells persisted in the circulation for 4 to 18 weeks after transfer in all patients, and at 6 months marked cells were found in lymphoid tissues (59). In subsequent studies, autologous HIV gag-specific $CD8^+$ CTL clones genetically marked with the LN retrovirus were infused (58). The infused CTLs were seen to accumulate adjacent to HIV-infected lymph nodes and transiently reduced the levels of circulating productively infected $CD4^+$ T cells. This decline was transient, likely due to a lack of $CD4^+$ help. However, the study did provide some rationale for pursuing this approach in conjunction with strategies to circumvent the requirement for CD4 cells.

VII. GENE MARKING OF MESENCHYMAL CELLS

Over the last few years there has been increasing interest in the use of mesenchymal cells to treat patients with genetic disorders affecting mesenchymal tissues, including bone, cartilage, and muscle. To evaluate this therapeutic option, Horwitz and colleagues infused gene marked, donor marrow-derived mesenchymal cells to treat 6 children who had undergone standard bone marrow transplantation for severe osteogenesis imperfecta (62). The marking component of this study showed that the mesenchymal cells could engraft in bone, skin, and marrow stroma (62). Of interest, the marker gene could not be detected in 1 patient likely due to an immune response directed at the *neo* gene (62).

VIII. IMPROVING MARKER STUDIES

A. Marker Gene

Most of the clinical studies reported to date have used *neo* as the reporter gene. This marker has the advantage that its safety has been widely studied in many different animal models, and it can be detected both phenotypically and genotypically. In gene marking the concern is that the activity of neomycin phosphotransferase (the product of the *neo* gene) is also likely to phosphorylate cellular proteins and may thereby modify the growth or differentiation of cells expressing the gene. In vitro, this effect has been observed in the HL60 cell line (63). There is evidence that a similar phenomenon occurs in vivo. There is the almost uniform observation that the proportion of cells positive for the neo marker gene in circulation is 0.5 to 1 log below the number of cells that are positive as determined by in vitro colony assays in the presence of the neomycin analog G418 (14). It may be that *neo* expression retards progenitor growth and differentiation in vivo, thus rapidly diminishing the numbers of *neo* progeny detectable. It is also possible that the expression of the transgene (i.e., *neo*) by the transduced cell results in an immune response that eliminates the transduced population. For some gene products, such as the hygromycin-thymidine kinase (Hy-Tk) fusion protein, the potency of this immune response is such that the response is a rapid elimination of large numbers of transduced cells in less than 48 h (52). It is as yet unclear if so potent an immune response regularly occurs after *neo* transduction, because neo-positive cells can be detected for up to 9 years after infusion and neo-marked T cells can be readily expanded in vivo by appropriate antigenic stimulation (55,56). If the discrepancy between levels of marking in CFU and mature cells is a consequence of the immunogenicity of the marker signal, then the interpretation of certain marker studies may be rendered more difficult.

Because of the limitations of the *neo* gene as a marker, several alternatives have been proposed. Amongst the most widely used in studies are GFP and cell surface markers such as the truncated (low-affinity) receptor for nerve growth factor (dLNGFR) (64–66). Unfortunately, many of these have the same limitations as neo and there is concern that some of these proteins (e.g., GFP) may prove to be significantly immunogenic in vivo (67). Moreover, aberrant expression of cell surface molecules may lead to unwanted cell trafficking or harmful intercellular contacts, even if the cell surface molecule has been modified to preclude intracellular signaling. One such concern is highlighted in a murine model using the same truncated dLNGFR marker gene that had been used in clinical studies (66). As discussed in Section IX. A, this gene was introduced by a replication-incompetent murine retroviral vector into bone marrow cells, which were then transplanted into irradiated mice. Marrow was then harvested after 28 weeks, pooled, and transplanted into secondary irradiated mice. These recipients subsequently developed hematopoietic disorders, carrying the same leukemic clone with a single vector copy integrated into the transcription factor gene Evi1 (68), and it was suggested that the capacity of dLNGFR to produce aberrant signal transduction contributed to the oncogenic events.

Because of the above concerns that marker gene may be immunogenic, may affect cell growth, and may contribute to oncogenicity, the most ideal marker may in fact be a nonexpressed sequence (62). However, until an ''empty'' retroviral vector is validated, *neo* seems to be the most appropriate marker gene presently available for clinical use.

B. The Use of Alternative Vectors

Although a detailed discussion of the pros and cons of the various vector systems available in clinical use is beyond the scope of this review, Table 3 summarizes the advantages and disadvantages of each. For gene marking studies, in which stable transduction is required and in which all daughter cells should contain equal amounts of the marker gene, integrating vectors are required. To date, this has meant using Moloney murine leukemia virus (MoMuLV) (19,21,69). The main limitation of the Moloney-based vectors is that they can only effectively transduce actively dividing cells, a major limitation when human hematopoietic stem cells are the targets because too few of these cells are in cycle at any one time.

Because of these limitations, human and feline lentiviral vectors have attracted increasing attention as gene delivery systems (70,71). Lentiviral vectors have been reported to readily transduce hemopoietic progenitor cells (72). Because they form a more stable preintegration complex than MoMuLV, they are also able to infect quiescent subsets of primitive hemopoietic stem cells, such as the $CD34^+$, $CD38^-$, or $CD38^-$ lineage negative population, where they persist and integrate once these cells enter cycle (73). HIV can also efficiently infect terminally differentiated cells such as neurons (74), and in both types of cell, high levels of gene expression have been reported. Although lentiviral vectors have clear advantages over murine retroviruses, substantial technical and safety problems remain before they can enter general clinical usage.

IX. VECTOR SAFETY CONCERNS

Adverse publicity has sensitized investigators and the public alike to the potential dangers of gene transfer vectors. Every new therapeutic agent must, of course, be carefully monitored

Table 3 Advantages and Disadvantages of Vector Systems

Vector	Advantages	Disadvantages	Current uses
Murine retrovirus	Stable integration into dividing cells Minimal immunogenicity Stable packaging system	Low titer Only integrates in dividing cells Limited insert size Risk of silencing Risk of insertional mutagenesis	Marker studies Gene therapy approaches using hemopoietic stem cells of T cells (e.g, to treat immunodeficiency syndromes) Transduction tumor cell lines
Lentivirus	Integrates into dividing cells Expressed in nondividing cells Larger insert size than murine retroviruses	No stable packaging system Complex safety issues	No approved trials as yet
Self-inactivating lentiviral vectors (SIN-Lenti)	Incapable of replication posttransfection? Increased safety Stable packaging system	? safety concerns remain	No approved trials as yet
Adenovirus	Infects wide range cell types Infects nondividing cells High titers High level of expression Accepts 12–15 kb DNA inserts	Highly immunogenic Nonintegrating	Direct in vivo applications Transduction tumor cells
Adeno-associated virus (AAV)	Integrates into dividing cells Infects wide range cell types	No stable packaging cell line Very limited insert size	Gene therapy approaches cells targeting muscle, liver, and brain
Herpesvirus	High titers Transduces some target cells at high efficiency Accepts large DNA inserts	No packaging cell lines Nonintegrating May be cytotoxic to target cell	Transduction tumor cells Neurologic disorders
Liposomes and other physical methods using plasmid DNA	Easy to prepare in quantity Virtually unlimited size Limited immunogenicity	Inefficient entry into target cell Nonintegrating	Topical applications Transduction tumor cells

for adverse effects, and gene transfer vectors are no different. Beyond these general concerns, however, there are more specific issues that must be considered. We have already alluded to concerns about recombinational events following administration of lentiviral vectors, but insertional mutagenesis is a potential consequence of any integration event, regardless of the vector used (32). Although most integration events of the vector DNA presumably harmless, there is an unknown (albeit low) risk that, in some cases, the integration event may result in activation of neighboring gene, such as an oncogene, which could result in uncontrolled cell division or ''insertional mutagenesis.'' Because tumorigenesis is believed to be a multistep phenomenon, it is likely that an additional event would be required before a vector insertion at a given locus would result in tumor formation. The Food and Drug Administration (FDA) addressed these risks nearly 10 years ago when they requested lifelong (now 15 years) follow-up of all gene therapy subjects who participated in clinical trials, including genetic analysis of any tumors that may appear.

Two recent events have refocused attention on this risk. In a murine study, marrow stem cells transduced with a murine retroviral vector encoding a marker gene (truncated dLNGFR) used in some human studies (66) caused leukemia after serial transplantation of transduced stem cells in mice (68). Although the resulting leukemia had an integration site in the Evi-1 gene, this is not usually sufficient for transformation and it is possible that the truncated NGFR served as a cofactor. In addition, a child in a clinical gene therapy trial in France for the treatment of X-linked severe combine immunodeficiency (SCID) (75,76) developed γδT cell leukemia—a serious adverse event related to the retroviral vector gene therapy. The expanded T cells were monoclonal, with overexpression of the LMO-2 gene and integration of the provirus in the promoter element of this gene, suggesting that the vector insertion may have caused dysregulation of the LMO-2 gene. In normal cells, the LMO-2 gene (present on chromosome 11) is expressed during early stages of hematopoietic differentiation and appears critical for lymphoid and myeloid cell development (77). Further, in T cell ALL, the chromosomal translocation t(11;14) results in the joining of the T cell receptor D or J segments to the LMO-2 locus. This is probably the result of aberrant RAG-mediated V(D)J recombination, thereby

highlighting the multistep nature of the leukemogenic process (77). It is currently unclear whether the selection advantage conferred by the ADA transgene contributed to this event.

A. Safety of Gene Marking

Although these 2 reports are of concern, it is important to remember that there have been no reports of malignancies related to insertional mutangenesis in patients who have received gene marked cells. Our group has followed over 75 patients who have received gene marked marrow or T cells for up to 10 years. So far, none of these patients have shown any evidence for selective expansion of marked cells. Two reasons for this are (1) gene marking occurs at a substantially lower level than gene correction. Hence fewer cells are placed at risk, and the probability of 2 or more integration events in a single cell is statistically exceedingly remote; and (2) unlike correction of a genetic defect such as X-linked SCID, gene marking does not lead to selection for transduced cells in vivo or to hyperexpansion of the ''genetically corrected'' T cell population. Even without gene transfer, such hyperexpansion of T cells can lead to lymphoproliferation and malignancy (78). This event occurred even when as few as 0.01% of stem cells or T cells were expressing the CD40L transgene. In this case, the malignant T cell did *not* contain the CD40L transgene.

Gene marking studies are used in adults and children in an effort to improve new or established therapies for immediately life-threatening illnesses for which current therapies are unsatisfactory. As importantly, marking studies help to determine whether novel therapies are contributing to any adverse events, minimizing subsequent exposure of vulnerable subjects. As discussed in previous sections, gene marking has been an invaluable tool in validating therapies that have had a real therapeutic impact (55,79), and at present we believe the proven benefits continue to exceed the potential risks.

X. CONCLUSIONS

Since the early 1990s, clinical studies using marker gene transfer have resolved many issues concerning the biology of hematopoietic cells and their progeny and have also helped to validate new treatments for viral infections and malignancy. To date, the approach has been safe, and the use of more advanced vector systems should increase the knowledge that can be obtained from this powerful tool.

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45

Gene-based Vaccination Against Malignant Melanoma

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I. INTRODUCTION

Gene therapy is an emerging field for treating diseases by using DNA as the remedy. It can be used to treat systemic diseases and various organ disorders. The goal of gene therapy is to treat a specific disease process with the protein product of the introduced gene. This protein can be either used locally or systemically (e.g., clotting factors and hormones) (1).

An essential component of the revolution in molecular biology has been the arrival of transfer methods by transfection or transduction. In principal, 3 different ways of introducing genetic material consist of (1) viral/bacterial vectors; (2) calcium phopshate, DEAE-dextran, or liposomes; and (3) physical methods such as direct injection, electroporation, or the gene gun (2). Each method has its own inherent strengths and weaknesses, and is especially suitable for a particular application.

In 1987, Sanford and coworkers invented a new addition to the armentarium of gene delivery vehicles, which was called ''biolistic method of gene transfer'' (3). Other terms include ''particle bombardment'' and ''gene gun.'' In principal, the technique implies that DNA is coated onto 1- to 5-μm heavy metal particles (usually gold or tungsten) that were accelerated in the early days using gun powder, today using helium, to sufficient velocity to penetrate the target cells. Historically, the first cells to be penetrated were plant cells (3,4). Its most notable application was the production of the first transgenic crop (i.e., the transfection of maize) (5,6).

To design potential treatment strategies for gene therapy, it is relevant to discuss 2 general approaches: the in vivo approach and the ex vivo approach of gene transfer (Fig. 1). In the in vivo approach, the desired genes are introduced directly into the target organ, whereas in the ex vivo approach, the target cells are cultured from biopsy specimens and the desired gene is inserted while these cells are being propagated in tissue culture. The genetically modified target cells are grown in culture and eventually grafted back onto the donor. Both the in vivo and the ex vivo approaches have their relative advantages and disadvantages, which reflect their potential applications. The biggest advantage of the in vivo approach is that it is simple and direct. In the case of skin, it takes advantage of its easy accessibility. The biggest disadvantage is that expression of the desired gene is usually transient because the gene is introduced locally and only into a limited number of target cells. Generally, stem cells have not been successfully targeted using in vivo approaches with the potential exception of hematopoiesis and liposomal gene transfer (7,8). However, it should be noted that transient expression of the desired gene may be adequate for a variety of applications such as genetic vaccination.

A plethora of different techniques has been developed in gene therapy to enhance the uptake of DNA (containing the gene of interest) (2). Initially, chemical DNA transfection has used calcium chloride and DEAE-dextran to transfer genetic material followed by the use of cationic lipids or liposomes. Because of the low transfection efficiency and the substantial in vivo toxicity of cationic lipids, other methods of gene transfer have increasingly been developed to insert DNA into cells. Viral gene transfer has exploited the capability of recombinant viruses such as retrovirus, adenovirus, or adeno-associated virus to infect cells and efficiently transport the genetic material containing the gene of interest into the cells. In addition, 3 physical techniques have been developed to introduce DNA into target tissues (2). These physical techniques are similar in that they can directly introduce DNA into the target organ such as skin. Consequently, these techniques will generally be used for in vivo approaches. Beside particle bombardment, direct injection using a syringe and a small needle has been

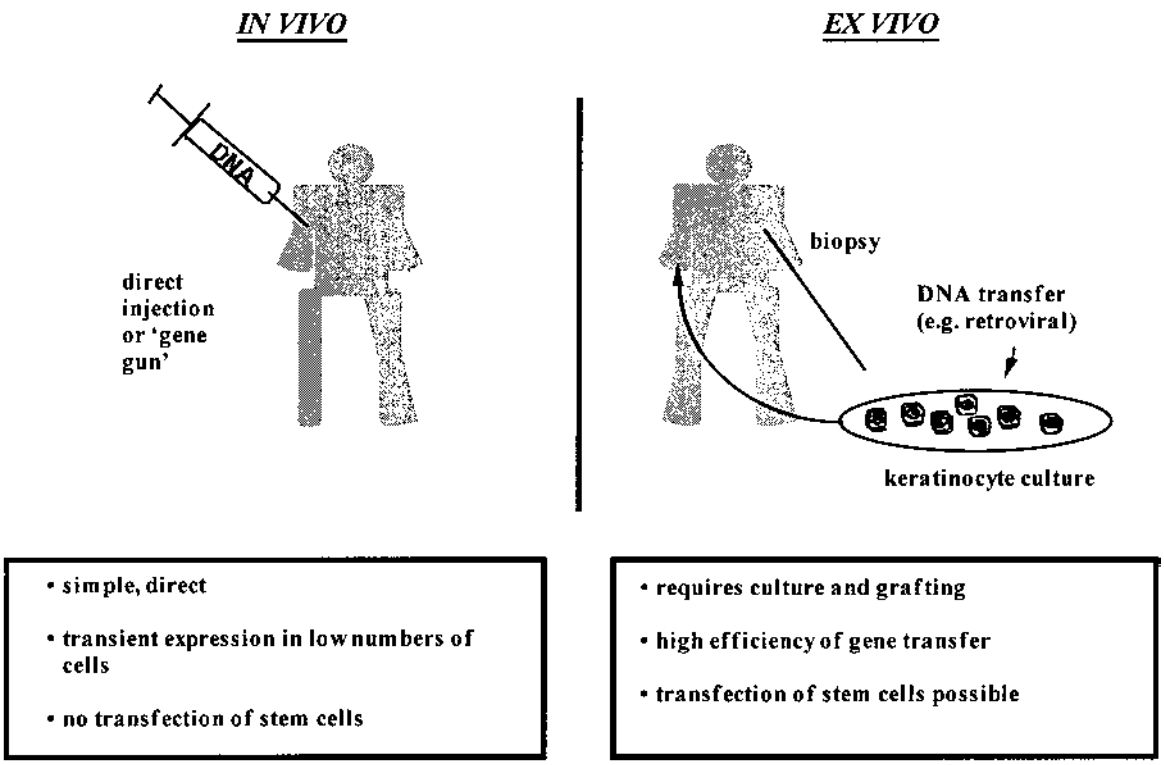


Figure 1 Schematic outline of in vivo and ex vivo gene transfer.

added as another alternative for gene transfer. Upon direct injection of DNA into skin or muscle, the DNA is taken up by the target cells and the desired genes are transiently expressed (9–12). In the case of injected muscle, the transgene can be detected for up to 1 year with some albeit low level of expression.

A variety of tissues has been successfully transfected using direct injection such as epidermis, muscle, thyroid, liver, lung, synovia, and melanoma (13–17). The exact mechanisms of how epithelial cells or muscle cells, the first tissue demonstrated to take up and express naked DNA, accomplish this is not yet clear, but there is evidence that a specialized transport process for small molecules, called potocytosis, may be involved (18,19). The existence of DNA-binding proteins on keratinocyte and muscle membranes is currently being investigated in the laboratory.

Feasibility studies have shown that plasmid DNA can also be expressed upon microinjection into the nucleus of mammalian cells (mouse fibroblast cell line LMTK$^-$), but not when injected into the cytoplasm (20). Furthermore, ultrasound has been demonstrated to allow gene transfer into mammalian cells in culture (21).

Alternative ways of introducing genetic material into the skin consist of various techniques to overcome the epidermal barrier that limits the delivery of plasmid DNA. Such methods comprise applying a pulsed electric field on topically applied plasmid DNA (22). A simpler way for gene transfer to the skin is constant puncturing using a device with oscillating fine needles (23). Topical application of plasmid DNA complexed to various cationic lipids has been demonstrated to allow gene transfer to the skin (24), although this approach has not worked for others. Topical application of plasmid DNA coding for the murine interleukin-10 gene to the scarified cornea has been shown to suppress an ongoing ocular inflammation caused by herpetic keratitis (25). The corneal route also proved effective in generating an immune response when expression plasmids coding for the herpes simplex glycoprotein B were applied (25).

The focus of this chapter is to review the progress made on genetic vaccination procedures using DNA to induce humoral and cytotoxic T cell responses.

II. BALLISTIC GENE TRANSFER

The pioneering studies by Sanford et al. demonstrated that a gene could be expressed in intact plant cells after biolistic transfer (3,4). The original gene gun as invented by Sanford and coworkers involved the placement of DNA-coated microprojectiles in an aqueous slurry on a small plastic bullet. This bullet was placed into a 22-caliber barrel in front of a gun powder cartridge. Upon firing, the cartridge propelled

a plastic bullet in the barrel until it was stopped by a solid plate. The plate had a central small hole that allowed the microprojectiles to continue their trajectory into the target tissue. Target organs or cells were placed in a chamber for bombardment. Subsequently, the gun powder device has been replaced because it created too much tissue damage and the velocity of the projectiles was not readily adjustable. Therefore, the helium device was developed as a collaboration of the Sanford and Johnston group, which used high-pressure helium that is restrained by a pierceable kapton disks. Another kapton disk, called macroprojectile, is positioned approximately 1 cm in front of the restraining membranes. The DNA-coated microprojectiles are placed in a dry state in front of this macroprojectile (Figs. 2A and B). Another centimeter in front of the macroprojectile a stopping screen is securely placed. Right in front of this stopping screen, a small orifice is put in contact with the tissue culture dish or the appropriate tissue. When the high-pressure helium gas is released by piercing the restraining membrane, a shock wave will be produced. This supersonic shock wave hits the macroprojectile, which is then launched against the stopping screen, allowing the microprojectiles to continue on into the target cells. An important technical feature is that the macroprojectiles seal off the chamber when they hit the stopping screen in order to protect the target tissue. All the single parts of the device are contained in a cylindrical chamber of the size of a soft drink can. In the chamber, where the microprojectiles are released, a vacuum is maintained in order to avoid deceleration by the air. The device is attached to a gas line and to a vacuum line, and an electric battery pack is used as power supply. This design is currently available from Biorad (PDS 1000/He; Hercules, California). Its design involves a restraining membrane that ruptures at the desired gas pressure. The distance the microprojectiles travel to the cells affects the transfection efficiency in several ways. First, especially for small particles, the velocity decreases with the travel distance. The velocity is an important variable with tissues explants, but not so with cultured cells. By placing the target cells closer to the stopping screen, the blast effect of the shock wave is increased. This effect is especially important for working with sensitive cell types. The area of bombardment increases with the distance from the stopping screen. On average, the bombarded area covers a 10-cm culture plate, when it is placed about 8 cm from the screen. The usual pressure of 25 mmHg (0.03 atm) is generally used to transfect most cultured cells. Second, studies on the plasmid delivery have determined that about 20 biologically active 6-kb plasmids can be carried on a single 0.5-mm microprojectile (26). Because commonly the microparticles are about 1 to 1.6 μm in size, considerably more copies of plasmid DNA can be loaded on 1 particle (27). However, the coating of individual particles is quite uneven. In general, tissues are bombarded with gold particles having a range of diameters from 1 to 5 μm. Tungsten particles are 3.9 μm in size. Microprojectiles are coated with DNA as described by Klein (4). Briefly, 25 μL of a gold or tungsten

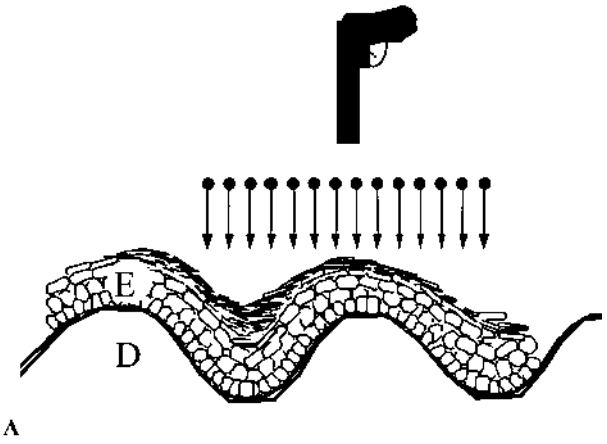
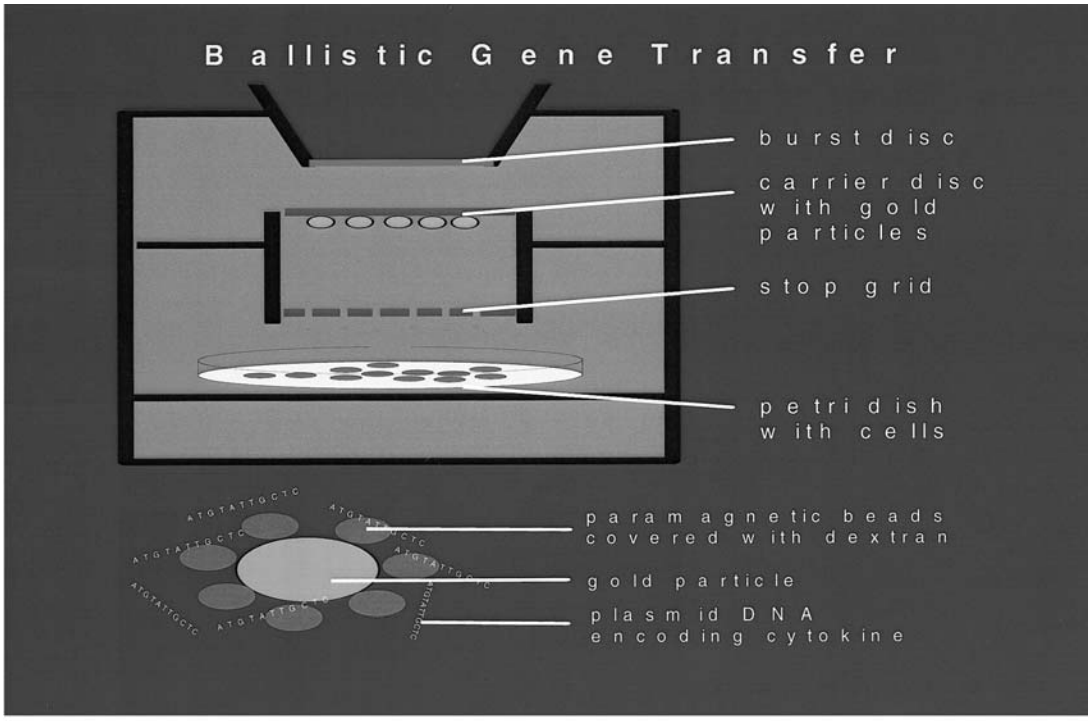


Figure 2 (A) Schematic of biolistic gene transfer. Colloidal gold particles are loaded with plasmid DNA encoding a cytokine and paramagnetic beads on the carrier disc. E, epidermis; D, dermis. (B) Detailed view of the biolistic process (see text for details). Pressure via helium gas is applied onto a burst disc causing its rupture and an acceleration of the loaded carrier disc. (C) The particle carrying disc is abruptly stopped at a stopping grid, causing a sudden release of the paramagnetic gold particles, which will subsequently hit the cells maintained in the petri culture disk at the bottom of the apparatus. Gold particles coated with paramagnetic particles and the plasmid DNA encoding human recombinant IL-7 or IL-12 will hit the tumor cells and pass the nucleus packed with chromatin. Because of the charge of the nuclear chromatin, the passing gold particles will lose most of their coated paramagnetic beads and plasmid DNA. Subsequently, cells hit are easily recovered by magnetic separation and selected for live cells, if necessary, by attachment to culture dishes. See the color insert for color version of parts (B) and (C) of this figure.

suspension are mixed with 25 μL of 2.5 M $CaCl_2$ and 5 μl of 1 M spermidine. After 10 min of incubation, the microprojectiles are pelleted and the supernatant is removed. The pellet is washed once in 70% ethanol prior to resuspension in 100% ethanol. The DNA-coated particles are spread onto macrocarrier discs and the ethanol is allowed to evaporate prior to loading the gun. Further refinement of the particle homogenicity will improve the DNA coating and will potentially lead to higher numbers of intact plasmid copies being delivered into the cells.

In the meantime, Agracetus (Madison, WI) has designed a similar device called ''Accell'' (28,29), which uses a large electrical discharge to vaporize a water droplet to create the impelling shock wave. Other adopted versions have been constructed for application in very sensitive tissues such as soft plants or seedlings as a handheld apparatus (''HandGun'' or ''Blowpipe'') (30) or airdriven (pneumatic) devices (31,32).



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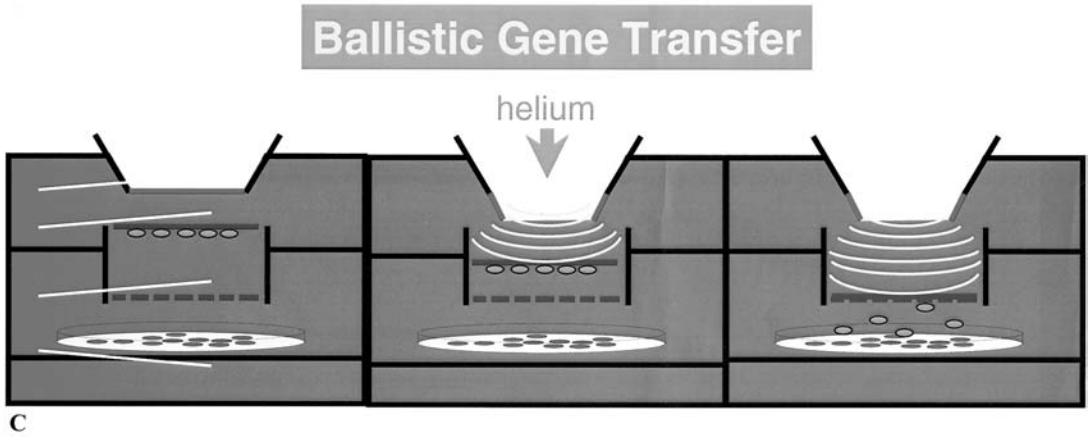

C

Figure 2 Continued.

III. GENE TRANSFER IN MELANOMA TREATMENT

A. Clinical Background

Malignant melanoma, the most aggressive skin cancer with rapidly rising incidence over recent decades, may be curable when resected at an early stage. In its metastatic stage, melanoma is highly resistant to standard forms of therapy (e.g., surgery, chemotherapy) and causes substantial mortality (33–35).

Given the putative role of the host immune system, and particularly the cell-mediated immunity in the pathogenesis of melanoma and in containing tumor growth, many attempts have been made to amplify host antitumor immune responses, such as regional or systemic administration of a variety of biological response modifiers (e.g., BCG, immunostimulatory cytokines, IL-2, IFN-α) and the use of various vaccines consisting of intact tumor cells/cell extracts (36–39). Although these treatment modalities induced impressive tumor regression in a subset of selected patients with metastatic melanomas, overall results were disappointing. Thus, it is imperative that new approaches—therapeutically effective but low toxic—are continued to be explored.

B. Melanoma and Tumor Immunology

Melanoma is a malignant tumor of neuroectodermal origin with an increasing incidence and mortality. It needs to be detected and eliminated early because melanoma is characterized by its high resistance to conventional therapies, including surgery and chemotherapy (33,40,41). However, melanoma is supposed to be one of the most immunogenic tumors, which is demonstrated by tumor infiltrating lymphocytes (TIL) destroying melanoma cells (36,38). This may also be responsible for the occurence of spontaneous partial or complete melanoma regression and for the concomitant destruction of melanocytes in benign lesions, leading to clinical phenomena such as halo nevi, uveitis, and vitiligo in melanoma patients.

It is generally accepted that the spontaneous generation of cancer cells is a common event, and that the immune system assures a strict surveillance with the detection and elimination of these cells. To fight cancer, the idea to use the destructive power of immunologic reactions is easily visualized in autoimmune diseases and by the rejection of allografts in transplantation medicine. A number of clinical observations in human malignant melanoma suggest a particularly vigorous immune response (36,38,42). In recent years, it became more and more clear that T lymphocytes may play a critical role in antitumor immune responses and surveillance (36,43). Furthermore, CD8+ T lymphocytes derived from melanoma lesions or the peripheral blood were shown to be capable to mediate impressive tumor regressions in vivo (44,45). The availability and further characterization of such tumor-specific T cell clones in the recent years led to the identification of several melanoma-associated antigens [reviewed in (43)].

Recent progress in molecular biology (particularly recombinant DNA technology) and in gene transfer techniques have stimulated considerable interest in the development of gene therapy for treatment of cancers. One of the most popular approaches currently being developed is so-called immunogene-based therapy, which has thus far involved a large set of cloned functional genes coding for immunostimulatory cytokines and other molecules such as costimulatory molecules (B7 family), adhesion molecules (ICAM-1), chemokines, or foreign MHC antigens. Based on successful animal studies, first clinical trials of using this novel approach, particularly with cytokine gene transfer therapy to fight melanoma tumors, have been worldwide conducted over the last 6 years. Another novel approach to tumor immunotherapy is called genetic immunization, also known as DNA or polynucleotide, immunization, which takes advantage of recently cloned genes encoding melanoma-associated antigens recognized by cytotoxic T lymphocytes (CTLs). This approach aims to enhance antigen-specific immune responses of the host to their own tumors by their gene products and is still at very early clinical stage. Furthermore, first treatment approaches using oligosensenucleotides and suicide genes have been developed and are discussed. This chapter reviews these novel immunological strategies.

IV. PRECLINICAL STUDIES

A. Genetic Immunization

The promising results using ballistic transfer techniques have led to the speculation that this process of DNA transfer could be employed in the generation of antibodies and gene therapy. In this regard, DNA technology seems superior to conventional protein vaccination considering economical and logistic aspects (availability, stability, storage). The concept behind genetic immunization is a simple one: genes encoding antigens are cloned into a plasmid with an appropriate promotor, and the plasmid DNA is administrated to the vaccine recipient. The DNA is taken up by host cells and the gene is expressed. The resultant ''foreign'' protein is produced within the host cell, then processed and presented appropriately to the immune system. This may lead to the induction of a specific CTL response through the MHC class I-restricted pathway. Concurrently, proteins are released extracellularly. It is believed that this exogenously released antigen primes the induction of a humoral response, as well as a helper T lymphocyte (Th) response via MHC class II-restricted antigen presentation by antigen-presenting cells (APCs) that have taken up the foreign antigen. The mechanisms of antigen processing and presentation to T lymphocytes are only partly understood. Class I MHC molecules present on the surface of antigen-producing myocytes or keratinocytes are probably sensitized with antigenic peptides derived from the antigen that can engage the T cell receptor. Humoral immune responses may be explained following the secretion of antigen from transfected somatic cells or by the release of antigen from lytic cells (46,47). Exogenous proteins released by this fashion could be taken up and presented to CD4-positive T cells by APCs in the draining lymph nodes. The presentation of genetically produced antigens to CD8-positive CTL is less clear. Besides the scenario that transfected nonprofessional cells such as keratinocytes present the antigen directly, antigens could be taken up by APCs via phagocytosis and gain access to the MHC class I pathway leading to the induction of antigen-specific CTL-mediated immunity (48–50).

In addition to the therapeutic antigen immunstimulatory cytokines such as GM-CSF, IL-1 or IL-12 in DNA form can be used as a vaccine cocktail (51–56). At least in some cases, the coexpression of costimulatory molecules such as B7.1 has further enhanced the immune response (57). Alternatively, the immunization can be performed with the complete expression library of pathogenic organisms (58,59) leading to the expression of all proteins coded by the infectious agent's DNA independent of their immunogenicity.

Using particle bombardment, a polyclonal immune response against various pathogens such as Ebola virus (60), hepatitis B and C (61–64), herpes simplex (65,66), malaria (51,67), mycoplasma (58,68), papillomavirus (69), prions (70), rotavirus (71), and tuberculosis (72,73) has been reported. Two diseases with a major impact on socioeconomic health served as a model: influenza and HIV. Pioneering work has been performed by Johnston et al. and Liu et al., who

demonstrated protection against heterologous strains of influenza (74–79). However, therapeutic and prophylactic vaccines against HIV have represented a great challenge for researchers. Several lines of progress have been made in mice (80–85) and nonhuman primates (86–88).

Various researchers have demonstrated the dependence of the immune response from the method and route of DNA administration. More specifically, saline injections of DNA vaccines into the skin have revealed a Th1-type antibody response, whereas gene gun immunizations produced a predominantly Th2-type immune response (i.e., IgG1) (85,89,90). In addition, the nature of the antigen also seemed to influence the type of immune response (90). The elicitation of a Th1-type immune response following the biolistic approach has been achieved by codelivery of vectors expressing IL-2, IL-7, or IL-12 (85). Various other factors such as the time period between immunizations have been identified to augment the Th1-type immunity (85). Direct comparison of the epidermal vs. the intramuscular route suggested a higher rate of seroconversion and higher antibody titers following epidermal immunization (29,91). The importance of APCs for the generation of an immune response has been clearly demonstrated in chimeric mice grafted with partially MHC-matched spleen cells (49,50) and in studies where direct transfection of dendritic cells with plasmid DNA has led to an effective immunization (48). This method of generating CTLs would alleviate the need of expanding and loading dendritic cells in culture prior to reinfusion into the host. Other adjuvants such as immunstimulatory CpG motifs may offer additional benefit (92,93). Furthermore, the presence of muscle cells following intramuscular injection of plasmid DNA has not been found necessary for effective immunizations (94). In contrast, the elicitation of antibody and CTL responses was dependent on the presence of the injected skin sites for at least 48 h (94,95). Further studies have shown that injected skin, which was transplanted to naive mice up to 24 h postinjection, could elicit a primary immune response (95). Variation of the delivery concept have included biodegradable microspheres showing an enhanced immunization potential compared with naked DNA (96). Successful immunization using plasmid DNA has also been shown in the female genital tract where vaginal mucosa expressed the introduced gene producing high levels of secretory IgA antibodies that were not consistently produced by other routes (97).

This section summarizes the evolutional steps of the particle bombardment technology and attempts to present some of the experimental data that has been obtained in various cell and tissue systems. The overall importance of this relatively atraumatic technology lies in the ease of inducing an effective DNA vaccination. However, the preferential Th2-type of the immune reaction needs to be kept in mind when considering potential target diseases. Based on recent experiments where protective immunity has been achieved in various animal models, this new technology using particle bombardment can be considered a potentially useful strategy for prophylactic or therapeutic vaccination of human.

The first report of DNA immunization that serves as antigen-specific tumor vaccine was made in early 1996. Zhai et al. (98) showed that inoculation of C57BL/6 (H-2^b) mice with recombinant adenovirus (Ad2CMV)-delivered plasmid DNA coding for human gp100 tumor antigen induced both anti-gp100 antibody and CTL responses. Importantly, immunization with Ad2CMV-gp100 protected mice from subsequent challenge with murine melanoma B16 in a $CD8^+$ T cell-dependent manner, indicating that vaccination generated anti-gp100 reactive T cells that were predominantly $CD8^+$ and responsible for tumor protection. Shortly after, several studies demonstrated protective tumor immunity in the B16-C57BL/6 mouse melanoma model by human gp100 DNA inoculation (99,100), independent of the use of different gene delivery vectors such as liposome or gene gun. In most cases, tumor protection appeared to be mediated by a specific CTL response because (1) mice deficient in MHC class I but not II were not protected by DNA vaccination, and (2) adoptive transfer of lymphocytes but not serum isolated from vaccinated mice that had rejected a previous B16 challenge mediated a dose-dependent protection of nonvaccinated recipients.

One important feature of the above-cited studies is that the protective immune responses to tumor challenge was induced by using xenogeneic DNA immunization; however, it is unclear whether immune tolerance to the autoantigen can be broken by this novel vaccine. To address this point, Wagner et al. (101) constructed a murine form of gp100-encoding plasmid DNA and then administrated to DBA/2 mice (H-2^d), another animal model. They observed that intracutaneous administration of murine gp100 DNA protected more than 50% of mice against a subsequent challenge with $gp100^{high}$-expressing M3-7 melanoma cells. Depletion of CD4- and CD8-positive T lymphocytes completely abrogated the protective effect. Thus, genetic vaccination with autoantigen enables efficient induction of antigen-specific, T cell-mediated immunity resulting in antitumor reactivity in vivo.

To explore the immunotherapeutic potential of other tumor antigen-encoding genes, Steitz et al. (102) examined the effect of TRP2 DNA vaccination. They found that immunization of C57BL/6 mice with both autologous murine and xenogenic human TRP2 DNA provided significant protection against metastatic growth of B16 melanoma in lung, with the xenogene displaying more pronounced protective effect and resulting in vitiligo-like coat depigmentation as a sign of autoimmune-mediated destruction of melanocytes. Interestingly, in this study, induction of protective immunity was found associated with both TRP2-reactive antibodies and $CD8^+$ T cells.

Furthermore, immunization with recombinant adenovirus was more effective than that using gene gun. In another study by Mendiratta et al. (103), some level of protective effect of genetic immunization with murine TRP2 was also detected. In this studies, however, a combination of murine TRP2 with human gp100 genetic vaccines had a synergistic effect and caused tumor rejection in 100% of the immunized mice. This suggests that polyimmunization with a mixture of tumor differentiation antigens may be a decisive strategy for a successful therapeutic vaccination. Taken together, these preclinical

studies have shown feasibility, safety, and great potential for the use of genetic immunization against melanomas. Therefore, further studies are warranted to define factors affecting the efficacy of a genetic vaccine, such as the vector system chosen, the dose of immunization reagents, the route and/or frequency of immunizations, as well as the use of adjuvant.

B. Genetic Targeting of Melanoma Cells

Targeted vectors will be necessary for many gene therapy applications. To target retroviruses to melanomas, a single-chain variable fragment antibody (scFv) directed against the surface glycoprotein high-molecular-weight melanoma-associated antigen (HMW-MAA) was fused to to the amphotropic murine leukemia virus envelope (104). The modified viruses bound only to HMW-MAA-expressing cells. Following attachment to HMW-MAA, MMP cleavage of the envelope at the melanoma cell surface removed the scFv and an introduced proline-rich hinge, allowing infection. Complexing of targeted retroviruses special liposomes greatly increased their efficiency without affecting their target cell specificity. In a cell mixture, 40% of HMW-MAA-positive cells but less than 0.01% of HMW-MAA-negative cells were infected. The authors concluded that this approach can therefore produce efficient, targeted retroviruses suitable for in vivo gene delivery and should allow specific gene delivery to many human cell types by inclusion of different scFv and protease combinations (104).

C. Grafting Primary Human T Lymphocytes with Cancer-specific Chimeric Single-chain and 2-chain TCR

Primary human activated T lymphocytes were genetically grafted with chimeric T cell receptors (TCRs) (105). Three domain single-chain (sc-) TCRs as well as 2-chain (tc-) TCR gene constructs were derived from the MAGE-A1–specific cytotoxic human T cell (CTL) clone 82/30, and linked to the CD3-zeta signaling element. Chimeric TCR alpha and beta receptor genes were structurally designed to prevent pairing with endogenous TCR alpha and beta chains in order to prevent the generation of unpredictable immune specificities. After transduction of polyclonally activated human peripheral blood lymphocytes with retroviral vectors harboring the chimeric receptor genes, genetically engineered cells specifically recognized and responded to MAGE-A1+/HLA-A1+ cells. Importantly, each type of transduced T lymphocytes that bound specifically to peptide/MHC complexes also showed specific antitumor reactivity as well as lymphokine production. The authors concluded that genetically engineered primary human T lymphocytes expressing chimeric sc- or tc-TCR therefore hold promise for disease-specific therapies (105).

V. IMMUNOGENE-BASED THERAPY—THE CONCEPT

Cytokines exert important functions in the modulation of host immune responses and display great therapeutic potential for cancer treatment when administrated systemically at high dose. These immune modulators have certainly gained considerable attention in the development of gene-based immunotherapy. One of the first reports, from Tepper et al. (106), showed that J558L plasmocytoma cells genetically modified with IL-4 gene were rapidly rejected after inoculation in syngeneic animal host. Since then, numerous cytokines have demonstrated the ability to reduce tumorigenicity when delivered in such a mode, these include IL-1, IL-2, IL-3, IL-7, IL-12, IFN-γ, TNF-α, or GM-CSF [reviewed in (107,108]). Importantly, in many instances, cytokine gene-modified tumor cells were also capable of stimulating a systemic protective antitumor immunity because vaccination of animals with gene-modified tumor cells protected them against a subsequent challenge of parental, unmodified cells at a distant site, and in some cases, even eliminated a preexisting tumor. Protection was often associated with T cell-mediated responses, whenever analyzed. Because several studies have used poorly or nonimmunogenic tumor models, it was suggested that the protective responses elicited were solely attributed to the transduced cytokine.

The conceptional considerations of using gene transfer as a means of immune modulation include (1) gene (cytokines, chemokines) transfer into the tumor cell, resulting in sustained local release may avoid apparent systemic effects of toxicity (Fig. 3), while producing dramatic local inflammation and immune activation either by transduced cytokines/chemokines, or through second cytokine secretion from activated APC such as dendritic cell (DC) or activated $CD4^+$ T cell; and (2) introduction of genes encoding costimulatory molecules (B7 family), adhesion molecules (ICAM-1), or foreign MHC into tumor cells may render them more immunogenic in terms of immune stimulation and/or T cell activation. Two major strategies are currently being developed for gene transfer—in vivo direct introduction of gene materials into the target cells and ex vivo gene transfer into explanted cells that are then reimplanted to the patient, by virtue of recombinant viruses or via various physical methods such as gene gun or liposomes. Both approaches have been applied to cancer patients with the use of various cytokine genes or allogeneic/xenogeneic MHC class I genes.

A. Ex Vivo Cytokine Gene Transfer—Clinical Phase I/II Trials

The apparent success in animal studies has fueled considerable interest in using cytokine gene-modified cell vaccines to treat cancer patients. Various approaches were used employing autologous, allogenic, mixtures of both, and xeniogenic cell systems. Progress and problems were recently reviewed (109).

1. Cytokine Gene Transfer into Autologous Tumor Cells

We have vaccinated a total of 16 patients with advanced melanomas in 2 successive phase I trials, one using autologous IL-7-gene-transduced tumor cells in 10 patients (110) and the other using IL-12-transduced tumor cells in 6 patients (111).

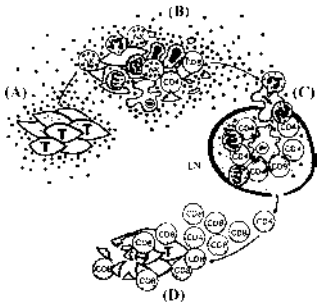

Figure 3 Rationale of cytokine gene transfer. Cytokine–gene transfer into tumor cells, causes a local secretion of cytokines (A) leading to an influx of immunological effector cells, such as T lymphocytes, NK cells, and DCs, and destruction of transduced tumor cells and inflammation (B). Destroyed tumor cells are taken up by antigen-presenting cells (APCs), which will migrate to the lymph node (LN) (C). After induction and expansion of tumor-specific T cells in the lymph node, tumor-reactive T cells of CD4- and CD8-phenotype will enter the circulation and destroy any (micro-) metastases detected at distant locations.

Immunizations were performed in weeks 1, 2, 3, and 6 by subcutaneous (s.c.) injection with total numbers of 5×10^6 to 3×10^7 modified tumor cells. In both trials, treatments were well tolerated and no major toxicity induced, except for mild fever and flulike symptoms in some patients vaccinated with IL-12-secreting tumor cells. Although no major clinical responses (complete remission: CR; and partial remission: PR) were achieved in any trial after the fourth vaccination, in IL-7-gene trial (110) 4 patients showed stable disease (SD) and 2 a mixed response (MR), whereas in IL-12 gene trial (111), 3 patients showed stabilization of the disease with 2 still alive for 10+ months. One experienced a mixed response with regression of some cutaneous metastases over 3 months. An extensive immunological evaluation was performed after the fourth vaccination. In the IL-7 gene trial, 4 patients showed increased (NK) cell activity and 7 showing an increased lymphokine activated killer (LAK) response upon vaccination (112). In 3 of 7 patients evaluated, the frequency of blood tumor-reactive CTLp increased between 2.6- and 28-fold. Of interest, all patients showing increased CTL responses were confined to clinical responders: 2 with a mixed response and 1 with a SD, implying a role of CTL in controlling tumor growth. Moreover, the magnitude of T cell responses induced was highly associated with patient's Karnofsky index and DTH reaction before vaccination, suggesting that patients with minimal tumor load or minimal residual disease may preferentially benefit from such a vaccine (110). In the IL-12 gene trial (111), vaccination induced DTH reactivity against autologous tumor cells in 2 patients, with 1 showing a heavy infiltrate of $CD4^+$ and $CD8^+$ T cells in regressing metastasis. The frequency of blood tumor-reactive CTLp was increased (up to 15-fold) in 2 patients after immunization. Furthermore, an analysis of both cohorts of patients demonstrated a significant increase of unspecfic NK and LAK activitity in patien's peripheral blood after vaccination compared with preimmunization (112).

Palmer et al. (113) conducted a phase II trial to investigate the biological effect of an autologous IL-2-secreting tumor cell vaccine introduced by retroviral gene transfer in 12 melanoma patients. Patients were treated for 1, 2, or 3 times with 10^7 modified tumor cells. No complications were observed. Three patients had stable disease for 7 to 15+ months, 1 for 17 weeks, the latter developed antitumor DTH after the first vaccination. In 4 patients, including 3 with 7 to 15+ months disease stabilization, the CTL responses to autologous tumor cells were increased upon vaccination.

Groups from Vienna, Freiburg, and Würzburg performed a phase I trial to evaluate the safety and tolerability of repeated skin injections of IL-2-transfected autologous melanoma cells into patients with advanced disease using a transferrin-mediated and adenovirus-enhanced transfection system (114). Fifteen patients received 2 to 8 skin vaccinations of either 3×10^6 (intradermal) or 1×10^7 (half intradermal, half subcutaneous) transfected melanoma cells per vaccination (secreting 140 to 17,060 biological response modifier program units of IL-$2/10^6$ cells/24 h). Overall, the vaccine was well tolerated. All patients displayed modest local reactions (erythema, induration, and pruritus) and some experienced flulike symptoms. Apart from newly appearing (4 of 14) and increasing (5 of 14) antiadenovirus and newly detectable antinuclear antibody titers (1 of 15), recipients developed de novo or exhibited increased melanoma cell-specific delayed-type hypersensitivity (DTH) reactions (8 of 15) and vitiligo (3 of 15) and showed signs of tumor regression (3 of 15). None of the patients exhibited complete or partial regressions (114).

2. Cytokine Gene Transfer into Allogeneic Tumor Cells

Obtaining sufficient number of autologous tumor cells for genetic manipulation is sometime problematic and is time consuming. Therefore, several studies have used well-characterized, allogeneic melanoma cell lines or fibroblasts as vehicles for cytokine delivery. Arienti et al. (115) and Belli et al. (116) first vaccinated 12 melanoma patients with 5 or 15×10^7 IL-2-gene-modified allogeneic melanoma cells. Three of 8 patients assessable experienced an MR. Two patients showed increased reactivity of specific CTL directed against tyrosinase and gp100 melanoma-associated antigens in postvaccination peripheral blood lymphocytes (PBL). Two additional patients showed an increased frequency of melanoma-specific CTLp in postvaccination PBL. The same group, in a subsequent study (117), tested IL-4-gene-transduced allogeneic melanoma cells in successive vaccinations in 12 melanoma patients. Both local and systemic toxicities were mild, consisting of transient fever and erythema, swelling and induration at the vaccination site. Two MR were recorded. Of 11 patients tested, antibodies and increased IFN-γ responses to allogeneic melanoma cells were documented in 2 and 7 patients, respectively, after vaccination. However, induction of a specific T

cell response to autologous tumor cells was obtained only in 1 of 6 cases studied. More recently, in a phase I/II study, Osanto et al. (118) applied an IL-2-secreting allogeneic melanoma cell line in 3 successive vaccinations (each 6×10^7 cells) to 33 melanoma patients. No major side effects were recorded. Two patients achieved complete or partial regression of subcutaneous metastases. Seven patients had protracted stabilization (4 to >46 months) of soft-tissue metastases, including 1 who developed vitiligo after vaccination. Immune responses to the vaccine could be detected in 67% of the 27 patients measured. In 2 of 5 patients, the frequency of antiautologous tumor CTLs was significantly increased by vaccination.

3. Cytokine Gene Transfer into a Mixture of Autologous and Allogeneic Tumor Cells

Differing from the above-cited studies using genetically modified tumor cells, Veelken et al. (119) in a pilot study applied IL-2-secreting allogeneic fibroblasts admixed with autologous tumor cells to 15 patients with advanced malignant tumors, including 6 melanoma patients. No major side effects were attributable to vaccines. In 2 melanoma patients, a dense infiltrate of both $CD4^+$ and $CD8^+$ T cells at vaccination sites was demonstrated. T cell lines generated from biopsies of these vaccination sites exhibited a dominant MHC class I-restricted cytotoxic activity against autologous tumor cells in vitro, and had identical V-D-J junctional sequence of TCR to that of infiltrating lymphocytes obtained from tumor sites of 1 patient. This suggests that the same CTL clone had infiltrated the tumor, circulated in the peripheral blood, and was amplified at the vaccination site (120).

At the Polish National Cancer Centers, a novel, mixed auto/allogeneic cellular melanoma vaccine modified with the IL-6 and the sIL-6R genes was designed (121). Preclinical studies in a mouse model demonstrated that the IL-6/sIL-6R-based vaccine is able to elicit efficient antitumor responses, mediated by CD8+ and NK cells, which resulted in inhibition of the tumor growth, metastases formation, and prolonged survival of the animals treated. Irradiation of vaccine cells does not only lead to their sterilization, but also causes increased secretion of exogenous IL-6 and sIL-6R. Between January 1996 and November 1999, the Polish group has vaccinated more than 100 metastatic melanoma patients (stage III and IV). Promising clinical results in 41 patients with measurable disease [22% CR (5/41) +PR (4/41), 32% SD (13/41)], and the evidence of immune responses in the vaccinated patients—including the induction of vitiligo—have prompted the group to design a prospective-randomized clinical phase III trial in stage III and IV melanoma patients, which was opened in 2000 to recruit 220 patients across Poland. The disease-free interval and 2-year overall survival will be compared with an observation arm as primary endpoints (121).

4. Cytokine Gene Transfer into Xenogeneic Tumor Cells

Based on the observation that immunodepression can be observed at the tumor site, clinical phase I clinical trials were initiated employing xenogeneic monkey fibroblasts (Vero cells) genetically engineered to produce human IL-2 (122,123). Various doses of xenogenic Vero cells were administered intratumorally in 8 patients with metastatic solid tumors. No severe adverse effect was observed. Obviously, Vero-IL-2 cells are rapidly eliminated from the organism and exogenous IL-2 mRNA could only be detected in the peripheral whole blood up to 48 h after Vero-IL-2 cell administration. A major finding of this trial concerns the 2 histological responses of 2 treated subcutaneous nodules not associated with an apparent clinical response. The relationship between local treatment and tumor regression was supported by replacement of tumor cells by inflammatory cells in regressing lesions and marked induction of T and NK cell-derived cytokines (IL-2, IL-4, IFN-γ) in posttherapeutic lesions analyzed 28 days after the start of Vero-IL-2 administration. Whether, the in vivo biological effect of immunostimulatory genes (i.e, IL-2-) may be potentiated by the xenogeneic rejection reaction needs further analysis.

B. In Vivo Cytokine Gene Transfer—The Rationale

In addition to ex vivo cytokine gene transfer, a substantial amount of work has recently focused on the direct implantation of the immunogenes in vivo. These have thus far involved the use of single cytokine or various combinations, such as cytokine/cytokine or cytokine/B7.1 costimulatory molecule. In different animal models, direct cytokine gene transfer into the tumor cells has been shown to improve the efficacy of the cytokine immunotherapy without the accompanying toxicity. Sun et al. (124) were the first to show that epidermal administration of human IL-6, TNF-α, and murine IFN-γ and IL-2 genes (by gene gun) could induce systemic antitumor effects in a renal carcinoma tumor model (Renca). Moreover, IFN-γ/IL-2 gene cotransfection resulted in eradication of preexisting tumors in 25% animals. Additional studies by Saffran et al. (125), using a murine IL-2 expression vector complexed with DMRIE/DOPE lipid, provided further evidence that intratumoral injection of IL-2 gene in Renca-bearing mice induced complete tumor regression that was mediated by $CD8^+$ T cells. Immunity was systemic, long lived, and Renca specific; further, adoptive transfer of splenocytes from treated mice protected naive mice against Renca tumor. These studies have shown an important feature for the direct in vivo gene delivery therapy: only combinations of cytokine/cytokine, cytokine/B7.1 costimulatory molecule, or cytokine/chemokine (IP-10 or MIG) could elicit a robust antitumor immunity.

1. In Vivo Cytokine Gene Transfer—Clinical Results

Results of first clinical trials at the level of feasibility studies have been appearing in the past few years, with most studies on metastatic melanomas employing the intratumoral administration of recombinant canarypox viruses—known as ALVAC—carrying cytokine genes or tumor-associated antigens (TAAs). ALVAC is an avipoxvirus derived from the licensed

Kanapox vaccine strain only capable to replicate in avian cells. IL-2-, IL-12- and GM-CSF-containing ALVAC-recombinant vaccines are currently been tested via direct intratumoral injection in clinical phase I/II studies in various European centers, including Zurich, Lausanne, and Leiden. Local inflammation, fever, cytokine serum levels, and histological infiltrate is highly dependent on inserted cytokine gene [reviewed in (126)]. In a small subset of patients, a tumor regression of injected nodules was observed without any effect on overall clinical disease progression. One of the major limitations is the strong anticanary immune response induced, which prevents repetitive administrations.

Insertion of genes or minigenes of TAAs were found to be of high scientific value in several studies. Motta and coworkers (127) have investigated the possible usefulness of ALVAC encoding the melanoma-associated Ag, Melan-A/MART-1 (MART-1). ALVAC MART-1-infected DCs were shown to express and able to process and present Melan-A/MART-1. Furthermore, infection by ALVAC induced apoptosis and cross-presentation of Ag occurred when uninfected DCs were cocultured with ALVAC MART-1-infected DCs. Uptake of apoptotic virally infected DCs by uninfected DCs, and subsequent expression of MART-1 in the latter, were verified by flow cytometry analysis, image cytometry, and confocal microscopy. Functional activity was monitored in vitro by the stimulation of a MART-1-specific cytotoxic T cell clone. The authors concluded that, overall, these data indicate that DCs infected with ALVAC may represent an efficient tool for inducing tumor-specific immunity, which can be exploited in clinical studies (127). Similarly, Chaux and colleagues (128) used a MAGE-A1-containing ALVAC construct to identify CTL epitopes by selecting naturally processed peptides. Therefore, monocyte-derived dendritic cells were infected with ALVAC containing the entire MAGE-A1 gene and were used to stimulate CD8+ T lymphocytes from the blood of individuals without cancer. Responder cell microcultures that specifically lysed autologous cells expressing MAGE-A1 were cloned using autologous stimulator cells, either transduced with a retrovirus coding for MAGE-A1 or infected with recombinant Yersinia-MAGE-A1 bacteria. The CTL clones were tested for their ability to lyse autologous cells, loaded with each a set of overlapping MAGE-A1 peptides. This strategy led to the identification of 5 new MAGE-A1 epitopes recognized by CTL clones on HLA-A3, -A28, -B53, -Cw2, and -Cw3 molecules. All these CTL clones recognized target cells expressing gene MAGE-A1 (128). A clinical evaluation of vaccination with a MAGE-1/MAGE-3 carrying ALVAC vector is presently being explored in Brussels (127).

VI. CONCLUSION AND PERSPECTIVES

Tumor immunology has made great progress in recent years. Recent animal studies have indicated that a potent protective immune response can be generated in vivo using cytokine gene-modified tumor cells. Locally secreted cytokines, such as IL-2, IL-4, IL-7, IL-12, or GM-CSF and others, from genetically modified tumor cells were shown to mediate tumor rejection and long-lived antitumor immunity. Based on these successful animal studies, various clinical protocols for the treatment of human cancer, predominantly using cytokine genemodifications, were initiated.

In summary, these clinical studies with both ex vivo and in vivo cytokine gene transfer therapy have demonstrated the feasibility, apparent safety, and low toxicity of these novel approaches. Treatment can enhance systemic antitumor cellular and/or humoral responses, or even result in objective tumor regression in a subset of patients with advanced melanoma. Because the magnitude of antitumor responses induced in patients were often correlated with a good clinical performance status, we assume these approaches would exert their most therapeutic effects in patients with low tumor loads. Nevertheless, one should keep in mind that standard therapy modalities such as chemotherapy needed decades before at least a few cancer entities could be cured. Until today, chemotherapy has not achieved a major benefit for patients with advanced malignant melanoma.

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