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The Basic Science of Gene Therapy

Richard C. Mulligan

The development over the past decade of methods for delivering genes to mammalian cells has stimulated great interest in the possibility of treating human disease by gene-based therapies. However, despite substantial progress, a number of key technical issues need to be resolved before gene therapy can be safely and effectively applied in the clinic. Future technological developments, particularly in the areas of gene delivery and cell transplantation, will be critical for the successful practice of gene therapy.

Ever since the development of recombinant DNA technology, the promise of the technology for dramatically improving the practice of medicine has been vigorously championed. Most of the advances affecting the clinical management of patients have involved either the development of new molecular techniques for the diagnosis of specific inherited and acquired diseases or the development of new therapeutic products made possible by the ability to engineer the overexpression of specific genes. Recombinant DNA technology has also produced the means for defining the roles of specific gene products in the pathogenesis of human disease. The ability to characterize disease in such molecular terms has already led to more precise and effective clinical interventions.

However, the idea underlying gene therapy—that human disease might be treated by transfer of genetic material into specific cells of a patient, rather than by conventional drugs—has yet to make its mark in medicine. Although the concept may appear to be elegantly straightforward and the most direct application of recombinant DNA technology, research has indicated that successful implementation of gene transfer in the clinic will require the coordinated development of a variety of new technologies and the establishment of unique interactions between investigators from divergent medical and basic science disciplines.

Although several reviews of gene therapy research have been published (*1*), few have focused on the technical issues that continue to impede the translation of preclinical studies of gene therapy into effective clinical protocols. In this review and commentary, I have attempted to define those issues and to suggest new areas of investigation that may help to resolve them.

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Development of Methods for Gene Delivery

The transduction of appropriate target cells represents the critical first step in gene therapy; consequently, the development of methods of gene transfer suitable for different forms of therapy has been a major focus of research (Table 1). The single common feature of these methods is the efficient delivery of genes into cells. In the case of retroviral vectors and adeno-associated virus vectors, the transferred DNA sequences are stably integrated into the chromosomal DNA of the target cell. These vectors have been considered most often for ex vivo gene therapy, which involves removal of the relevant target cells from the body, transduction of the cells in vitro, and subsequent reintroduction of the modified cells into the patient. All of the other methods of gene transfer result primarily in the introduction of DNA sequences into the nucleus in an unintegrated form. Those methods, which result in high, but transient, gene expression, have predominantly been considered for use in in vivo gene therapies, in which genetic material is directly transferred into cells and tissues of the patient. As discussed below, little is known about the fate and properties of DNA delivered to cells by these other methods.

Retroviral vectors. Interest in retrovirus-mediated gene transfer stems primarily from the ability of some vectors to stably transduce close to 100% of target cells.

Although retrovirally mediated gene transfer is ideal for many ex vivo applications of gene therapy, several features of the gene transfer method may limit its applicability, particularly with regard to in vivo therapies. First, retrovirus entry into cells is absolutely dependent on the existence of the appropriate viral receptor on the target cell. To provide a means of infecting most cells of interest, researchers have developed packaging cell lines capable of generating viruses of a variety of host ranges (*2*). Because the identities of most retroviral receptors are unknown, however, it has not been possible to determine the distribution of receptors in different cell types. Problems encountered in transducing specific cell types (such as hematopoietic stem cells) may be due, in part, to the lack of expression of appropriate viral receptors. Second, replication of the target cells is necessary for proviral integration to occur. Although previously it had been assumed that this requirement reflected the necessity for DNA synthesis, recent studies suggest that viral integration may depend on mitosis (*3*). Thus, successful gene transfer depends on the ability to induce proliferation of the target cell, at least for short periods of time.

Another problem is that the retroviral

particle is relatively labile in comparison to other viruses. Attempts to purify or concentrate retroviruses can lead to significant loss of infectivity, partly due to loss of the viral *env* product. In addition, amphotropic virus is rapidly inactivated in vivo in primates, presumably via a complement-mediated process (*4*). Both the instability of retrovirus particles and the inability of retroviruses to integrate in nonreplicating cells most likely account for the difficulties encountered in the use of retroviral vectors for transduction in vivo.

The single most important advance in the development of retroviruses as gene transfer vectors was the generation of specialized cell lines (termed "packaging cells") that permit the production of high titers of replication-defective recombinant virus, free of wild-type virus (*2*). In principle, since no genetic information for virus production is transferred from the packaging cells, transduced cells are unable to perpetuate an infection and spread virus to other cells. Nevertheless, several "outbreaks" of wild-type virus from recombinant virus–producing cell lines have been reported (*5*). Furthermore, in recent primate bone marrow transplantation experiments in which hematopoietic stem cells were transduced by stocks of recombinant retrovirus contaminated with replication-competent virus, several of the transplanted animals developed lymphomas (*6*). Attempts to understand the basis for the low level of encapsidation of virus protein–encoding transcripts observed in existing packaging cell lines may be particularly relevant to the design of improved cell lines.

Adenovirus vectors. These vectors are of interest because of their potential for in vivo gene delivery. Adenoviruses are capable of efficiently infecting nondividing cells and expressing large amounts of gene products. Furthermore, the virus particle is relatively stable and amenable to purification and concentration. In contrast to retroviruses, infection of permissive cells with adenovirus leads to cell lysis. Although integration of adenoviral DNA sequences into chromosomal DNA of the target cell can occur, particularly at high multiplicities of infection in nonpermissive cells, integration does not appear to be an integral part of the viral life cycle and is relatively inefficient.

The most critical advance in the development of adenovirus vectors was the finding by Shenk and co-workers that replication-defective adenoviruses lacking portions of the E1 region of the viral genome could be propagated by growth in cells engineered to express the E1 genes (*7*). Subsequent studies indicated that defective adenoviruses genomes carrying foreign DNA sequences could be propagated in the same way (*8*). Most of the adenovirus vectors currently in use carry deletions in the E1A-E1B and E3 regions of the viral genome. Expression of inserted sequences is usually promoted either by the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Although adenovirus vectors have been used extensively to produce large quantities of gene products in vitro (*8*), examination of their use in gene therapy has been limited. Preclinical studies with adenovirus vectors have involved the administration of concentrated virus to a variety of tissues and the assessment of subsequent vector-mediated gene expression (*9–14*). Studies of this type have demonstrated that efficient transduction of a significant number of cells in vivo could be achieved and that gene expression could persist for significant periods. Because there is little precedent for the prolonged persistence of unintegrated DNA, at least in cells in vitro, the mechanism underlying this phenomenon is of great interest. Of relevance is the possibility that existing adenovirus vectors are at least partially replication-competent. Although expression of the E1 region has been shown to affect the expression of other viral gene products necessary for replication (*15*), the requirement of E1 gene expression for viral replication does not appear to be absolute. The early characterization of E1-deficient adenoviruses demonstrated that at high multiplicities of infection, the E1 region was dispensable for replication (*7*). Other studies, however, have also demonstrated that even at low multiplicities of infection, a normal replicative cycle can occur, although more slowly (*16*).

These findings suggest that it is likely that a low level of replication of recombinant virus may occur in vivo and perhaps even be responsible for the persistence of gene expression in vivo that has recently been observed. Because the ability of E1-defective adenovirus genomes to replicate may depend on the expression of specific host gene products or the proliferative state of the cells (*17*), the capacity of adenovirus to replicate in vivo could be critically dependent on the nature of the target cell. Consequently, it is unclear whether most available animal models will accurately predict either the replicative capacity of adenovirus vectors or their capacity for persistent gene expression in specific human cells in vivo.

If replication of adenovirus vectors does occur in vivo, there may be cause for safety concerns. The adenovirus life cycle involves the temporal expression of a large number of gene products with diverse biological activities (*15*). In addition to the expression of genes that lead to the shutoff of host protein synthesis, a number of gene products involved in malignant transformation are also expressed. At least one viral structural protein expressed late in the replication cycle is specifically toxic to cells (*18*). In addition to such intrinsic toxicities, expression in transduced cells of the large number of viral proteins normally synthesized during the course of a productive adenovirus infection may lead to the induction of a powerful immune response directed against the cells. Such issues of toxicity in vivo have not yet been adequately evaluated.

Other viral vectors. Less well developed viral vector systems that may have application in gene therapy have been derived from adeno-associated virus (AAV), herpes virus, vaccinia virus, and several RNA viruses. AAVs are relatively small viruses, remarkably stable, and can infect human cells.

Table 1. Methods for delivering genes into mammalian cells and likely applications in gene therapy. (+), Major application; (+/−), some application; (−), little or no application; T, only transient expression; S, stable expression.

Method	Application in gene therapy		Transient (T) or stable (S) expression
	Ex vivo	In vivo	
Viral			
Retrovirus	+	?	S
Adenovirus	+/−	+	T
Adeno-associated virus (AAV)	+	?	S
Herpes virus	+/−	+	?
Vaccinia virus	+/−	+	T
Polio virus	+/−	+	T
Sindbis and other RNA viruses	+/−	+	T
Nonviral			
Ligand-DNA conjugates	−	+	T
Adenovirus-ligand-DNA conjugates	−	+	T
Lipofection	+/−	+	T
Direct injection of DNA	−	+	T
$CaPO_4$ precipitation	+/−	−	S

Integration of AAV genomes into host DNA (*19*) appears to be less efficient than retroviral integration and also less precise, as tandem viral genomes with slight deletions or rearrangements (or both) are often observed. An unusual property of wild-type AAV is that integration of the viral genomes frequently occurs within a small region of human chromosome 19 (*20*). However, because this region has been implicated in chromosomal rearrangements associated with chronic B cell leukemias (*21*), it is unclear, from the standpoint of safety, that such site-specific integration would offer any advantages over random integration. Furthermore, none of the vectors constructed to date seems to retain this property. A second property that has been attributed to AAV is the ability to integrate into nondividing cells. In light of the critical importance of this property (see below), it is surprising that no direct experimental evidence exists to support this claim.

Herpes virus vectors (*22*) may provide a unique strategy for persistence of foreign DNA in cells of the central nervous system (CNS). However, because of the complex regulation of viral replication, it has been difficult to generate stocks of recombinant virus that are completely incapable of replication. Although recently this has been achieved, it appears that even replication-incompetent virus can kill cells, presumably because a number of specific viral gene products that are still expressed in the infected cells are toxic (*23*). Current efforts are aimed at preventing the expression of such genes.

Finally, a number of viral vectors designed to replicate in target cells have been considered for specific applications in gene therapy where it is desirable to transiently express large amounts of gene products (for example, in immunization) (Table 1). These include vectors derived from vaccinia virus, poliovirus, and several other RNA viruses.

Nonviral methods of gene transfer. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. At present, interest is centered on methods that rely on receptor-mediated endocytic pathways for the uptake of DNA because such methods may permit the targeted delivery of genes to specific cell types in vivo. Receptor-mediated methods of gene transfer involve the generation of complexes between plasmid DNA and specific polypeptide ligands (*24*) that can be recognized by receptors on the cell surface. One of the problems with receptor-mediated uptake for gene delivery is that the endocytic vesicles formed during this process are usually transported to the lysosome, where the contents of the endosome are degraded. Consequently, effective delivery of DNA to cells via receptor-mediated endocytosis depends on escape of the DNA from the endosome during the course of its transport. Appreciating this fact, Birnstiel and co-workers have used either whole adenovirus or fusogenic peptides of the influenza HA gene product (*26*) to induce efficient disruption of DNA-containing endosomes. In both cases, the proportion of cells that expressed the transferred DNA sequences and the absolute levels of expression obtained were markedly higher than those achieved with ligand-DNA complexes alone. Woo and co-workers have confirmed this finding and further demonstrated the ability of adenovirus to enhance the targeted delivery of genes (*27*).

Although the results obtained with receptor-mediated methods of gene transfer are promising, the data on in vivo expression suggest that this method may permit only transient expression of genes; consequently, these methods may have only limited application. As with adenovirus vectors, the molecular basis for the persistence of gene expression in receptor-mediated gene transfer should be examined further.

Development of Preclinical Models of Gene Therapy

Ideally, preclinical studies of any potential therapy should involve both the development of suitable protocols for carrying out the key technical steps and an assessment of the therapeutic efficiency and safety in an appropriate model of the disease. Currently, preclinical studies are still focused primarily on establishing the technical feasibility of different forms of therapy. Significant progress has been made in a number of areas.

Diseases affecting hematopoietic cells. Most efforts have focused on hematopoietic stem cells because the transduction and transplantation of these cells would provide a means of ensuring a continuous supply of genetically modified hematopoietic cells during the lifetime of the patient. The transduction of stem cells has proven to be quite difficult, mainly because they are found only in small numbers in bone marrow and appear to be primarily quiescent. Efforts to optimize conditions for transduction have focused on examining ways to increase the proportion of stem cells in cycle. The greatest obstacle to the development of efficient transduction protocols has been the lack of available assays for quantifying stem cells. Although a number of in vitro colony assays have been developed for the quantitation of various hematopoietic progenitors, none appears to directly assay for the stem cell capable of reconstituting lethally irradiated recipients, the desired target cell for most gene therapies involving hematopoietic cells. In addition, the in vivo assay that has most often been used for quantitating stem cell numbers, the 14-day colony-forming unit–spleen (CFU-S) assay, does not directly measure cells with reconstitution activity (*28*) and therefore is not appropriate for evaluating stem cell transduction protocols.

The most reliable means of evaluating different transduction protocols has proven to be the examination of the mature hematopoietic cells from bone marrow transplant recipients engrafted for long periods with genetically modified cells. Such studies indicate that, without prior treatment of the stem cells, only a small proportion of the stem cell population can be transduced, by either virus supernatant infection or cocultivation of the cells with virus-producing cells. The most successful transduction protocols have involved the use of bone marrow cells from mice previously treated with the drug 5-fluorouracil (5-FU) and the addition of growth factors [in most cases interleukin-3 (IL-3) or IL-6, or both] during or before the cocultivation of stem cells with virus-producing cells (*29*). By means of such protocols and high-titer viruses, bone marrow transplant recipients have been reproducibly generated in which recombinant provirus is present in close to 100% of all hematopoietic cells. A variety of different genes have now been expressed in hematopoietic cells in vivo (*1, 30*).

Although early reports suggested that retroviral vectors that rely on the viral long terminal repeat (LTR) for the transcription of inserted sequences did not lead to sustained gene expression in hematopoietic cells in vivo, recent studies suggest that the viral LTR is active in most hematopoietic cells in vivo and will be generally useful for obtaining the constitutive expression of genes (*31*). Obtaining the regulated expression of transduced genes, however, has been more problematic. In experiments with the human β-globin gene, for example, retrovirally mediated transfer of the gene to hematopoietic stem cells does lead to erythroid-specific gene expression, but only at very low levels (approximately 1 to 2% of normal murine β-globin levels) (*32*). In addition, the efficiency of transduction of stem cells with β-globin gene–containing vectors has generally been low because of the low viral titers obtainable with most β-globin constructs. The finding that additional sequences located upstream of the β-globin gene (termed lcr sequences) are necessary for high levels of β-globin transcription (*33*) suggested a means for improving β-globin gene expression in retroviral vectors. However, incorporation of these sequences into retrovirus vectors in such a way that the resulting recombinant genome is efficiently packaged and trans-

ferred faithfully to cells has proven difficult. Although recent studies have reported the generation of such lcr-containing β-globin vectors and demonstrated that the inclusion of the lcr sequences can in some cases markedly increase the levels of β-globin expression (*34*), the vectors used have low titers and are probably not suitable for clinical use. The generation of high-titer viruses encoding the relevant lcr and β-globin gene sequences remains a serious technical obstacle to the treatment of diseases such as β thalassemia and sickle cell anemia by gene therapy.

Protocols for the transduction of dog and monkey hematopoietic stem cells have been developed and evaluated by means of the analysis of a relatively limited number of bone marrow transplant recipients reconstituted with genetically modified cells. These studies indicate that only a very small proportion of stem cells (~1%) can be transduced (*35*). Studies of the transduction of human hematopoietic stem cells have relied exclusively on in vitro assays that score for multipotent progenitor cells. Although a significant proportion (more than 50%) of long-term culture–initiating cells (LTC-ICs), the most primitive hematopoietic progenitor detectable in vitro, can be transduced (*36*), the relation between LTC-ICs and cells capable of reconstituting patients remains unclear.

Overall, progress toward the development of gene therapies involving hematopoietic cells continues to be impeded by our lack of knowledge concerning both the functional properties of stem cells and the biology of bone marrow transplantation itself. Although procedures for purifying stem cells with reconstitution capacity have recently been developed, they have been applied only in a limited fashion to studies of stem cell transduction (*37*). Consequently, most of the information available regarding the effects of various transduction protocols on the proliferation of stem cells is derived from indirect evidence. For example, although it is thought that both the pretreatment of donor mice with 5-FU and the culture of bone marrow cells with hematopoietic growth factors increase the proportion of stem cells in cycle, and therefore the proportion of cells susceptible to retroviral infection (*38*), there is little direct data to support this idea.

An alternative possibility is that such manipulations of cells influence the engraftment of transduced cells rather than their proliferative status. If, for example, the small proportion of cycling stem cells in bone marrow that are susceptible to retroviral infection normally compete with other cells for the engraftment of recipients, then the elimination or alteration of specific populations of cells by manipulation of the marrow may lead to selective engraftment with cycling cells. In such a case, the desired transduction of most of the mature hematopoietic cells present in reconstituted recipients could be achieved even though only a small proportion of stem cells were actually transduced. It might then make more sense to attempt to purify the small population of cycling cells that normally exist and to use them as targets for gene transfer and transplantation than to develop in vitro culture conditions or other manipulations that bring quiescent cells into cycle. We are testing this hypothesis. These issues underscore the need for a more extensive understanding of bone marrow transplantation, particularly the role of different cell populations involved in the engraftment process (*28*), in order to develop effective transduction protocols for hematopoietic cells.

Diseases of the liver and lung. In the case of liver-directed gene-therapies, both ex vivo and in vivo models of therapy have been investigated. Although progress has been made in the development of protocols for transducing hepatocytes for ex vivo therapies, technical problems continue to plague the transplantation of hepatocytes. Even under optimal conditions, injection of hepatocytes into the liver or spleen results in the engraftment of cell numbers representing only a small percentage (<10%) of the total number of cells in the liver (*39*). The importance of methods for ectopically grafting hepatocytes is being increasingly recognized (*40*).

A variety of experiments aimed at developing direct in vivo gene therapies for liver disease have been performed. In studies by Wu and co-workers with the Watanabe rabbit, transfer of complexes carrying a low density lipoprotein receptor (LDL-R) expression construct led to detectable but transient expression of LDL-R (*41*). For reasons not well understood, partial hepatectomies of recipients before administration of the complex resulted in prolonged expression in vivo (*42*). Studies by Perricaudet and coworkers have also established that adenovirus vectors can efficiently infect hepatocytes (*14*). Lastly, investigators have demonstrated the ability to transduce hepatocytes in vivo with retroviral vectors (*43*). Because of the great interest in liver-based therapies and the technical and clinical problems associated with ex vivo approaches, it is likely that efforts in the future will be directed primarily toward establishing in vivo gene therapies for the liver.

For the treatment of lung diseases, in vivo models of gene therapy have received primary consideration. Crystal and coworkers demonstrated the ability of adenovirus vectors to deliver several genes to the airway epithelium of the cotton rat, a well-studied model of adenovirus infection (*9, 12*). A large proportion of cells could be transduced, and expression persisted for 7 days in one study (*9*) and 42 days in another (*12*). Similar results have been obtained by other investigators with adenovirus vectors (*5*). Lipsome-mediated gene transfer has also been successfully used for delivery of genes to the airway epithelium (*44*). Using this method, Hyde *et al.* (*45*) delivered expression constructs for the cystic fibrosis transmembrane conductance regulator (CFTR) into lung epithelia and alveoli of mouse strains containing disruptions of the CFTR gene. Evidence for expression of the CFTR gene was obtained by RNA in situ hybridization and voltage clamping measurements of ion transport. The persistence of gene expression, however, was not examined. Finally, Gao *et al.* (*46*) reported the successful transduction of airway epithelial cells by using adenovirus-based receptor-mediated gene transfer and demonstrated expression for up to 1 week after transduction. Because the most serious technical limitation of the above approaches is that gene expression can be obtained only for short periods, it will be important to assess the consequences of repeated use of these methods.

Clotting disorders and other diseases involving circulating gene products. Preclinical studies in this area are aimed at the development of methods for delivering gene products to the circulation. For this application, the choice of target cells is dictated by a number of practical considerations, including the ease of isolation and culture of the cells, the half-life in vivo, the capacity of the cells for vector expression, and perhaps most importantly, their capacity to make contact with the circulation after transplantation. Although initially established cell lines were studied (*47*), research has more recently focused on the transplantation of primary cell types. Most progress has been made in the transplantation of transduced keratinocytes, myoblasts, and fibroblasts. It appears that many of the problems encountered with the transplantation of these cells were at least partially attributable to the use of vector-mediated expression in the transduced cells as the primary indicator of graft survival. The development of methods for independently assessing graft survival and gene expression has been critical for establishing that gene expression in vivo can be a significant problem and for providing a means of testing different vector constructs (*48*). A limited number of experiments now suggest that persistent gene expression in vivo after the transplantation of transduced cells is possible. In the case of keratinocytes, standard retrovirus vectors and well-established methods for transplantation have been applied successfully (*49*). Unfor-

tunately, while in some cases low levels of gene expression persist, no vector-encoded gene product has been efficiently delivered to the circulation for sustained periods. As several gene products synthesized endogenously by keratinocytes can be efficiently delivered to the circulation (*50*), the reasons for the problems with exogenous genes are unclear. Experiments with both myoblasts and fibroblasts have demonstrated long-term expression of transduced genes after transplantation. In the case of myoblasts, human factor IX was shown to be efficiently synthesized and delivered to the circulation for more than 6 months after injection of transduced primary cells into muscle (*51*). Expression of growth hormone has similarly been demonstrated with myoblast cell lines (*47*). In the case of fibroblasts, both long-term expression of β-glucuronidase (for more than 5 months) and correction of the lysosomal storage defect in β-glucuronidase–deficient mice have been achieved (*52*). In both the myoblast and the fibroblast studies, it was necessary to rely on vectors that contain nonviral, transcriptional sequences for promotion of gene expression because LTR-based vectors were inactive (*51, 52*).

In spite of progress in this area, the results to date should be considered as tentative. With myoblasts and fibroblasts, few of the parameters critical for successful transplantation have been rigorously established. It is unclear, for example, whether the age of the cells, their culture history, and the particular site of transplantation of the cells might not all be key parameters for success. In addition, the long-term fate of transplanted cells has not been examined in detail. It will be critical to more precisely quantitate the size and structure of the graft over time and to understand the migratory properties, if any, of the cells. Furthermore, the immune response to genetically engineered grafts may dictate both the type of cell that can be transplanted and the location for grafting. In cases where gene products are to be delivered to the circulation, a variety of issues regarding the contact of the transplanted cells with the circulation should be examined. The studies with keratinocytes also suggest that the efficient delivery of gene products into the circulation may depend at least in part on the nature of the gene product (*49*). Although the demonstration that transplanted myoblasts could efficiently deliver Factor IX to the circulation is encouraging, it is not known whether other gene products, particularly larger molecules like Factor VIII, can be delivered in the same way. The application of in vivo methods of gene delivery to the systemic production of gene products remains to be established.

Acquired diseases. The possibility that gene therapy might be applied to the treatment of acquired diseases is being investigated in a number of preclinical studies. Potential therapies for cancer have focused primarily on the genetic modification of either tumor-specific lymphocytes or tumor cells. Rosenberg and co-workers are attempting to use tumor-infiltrating lymphocytes (TILs) (*53*) to locally deliver large amounts of cytokines and other gene products with antitumor activity to sites of tumor in vivo in order to circumvent the toxicity associated with the systematic delivery of those gene products. One of the problems with the approach is that in spite of data indicating some clinical efficacy of TIL therapy (*53*), neither the capacity of TIL to traffic to tumor in vivo nor the mechanism of antitumor activity of TIL has been adequately established. In addition, it is unclear whether the constitutive expression of cytokines and other gene products by TIL will be beneficial, since the expression of many gene products of T cells is tightly regulated.

Several studies involving the transduction of tumor cells have attempted to enhance the ability of the cells to stimulate a tumor-specific immune response. As a first step, it has been demonstrated that the expression of specific cytokines and other gene products can cause the rejection of tumors that would otherwise grow progressively in the host (*50*). In some cases, transduced tumor cells can promote the rejection of a subsequent challenge of nontransduced tumor cells, or preexisting tumor (*50*).

We have recently compared the ability of a number of cytokines and other gene products, including many of the gene products previously shown to have antitumor activity, to stimulate antitumor responses (*54*). Our work suggests that previous studies that identified specific gene products as having antitumor activity may need to be interpreted with caution because the immunogenicity of the tumors used in those studies makes it difficult to define the antitumor activity of the gene products examined. By using a relatively nonimmunogenic tumor model, however, we identified specific gene products that induce levels of antitumor immunity that cannot be achieved with tumor cells alone. These studies form the basis of clinical protocols that will investigate the use of irradiated tumor cells expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), the most potent of the molecules examined, as therapeutic vaccines for the treatment of a variety of different cancers. Other studies using live or irradiated tumor cells will examine the activity of a number of other gene products (*5*).

In addition to forming the basis for therapeutic cancer vaccines, the ability of tumor cells expressing specific gene products to stimulate antitumor responses may also facilitate the isolation of tumor-specific T cells from cancer patients, which has proven to be quite difficult (*55*). Such cells might be used both in adoptive transfer therapies and as reagents for the isolation of new tumor-specific antigens (*56*). Finally, the results obtained with transduced tumor cells suggest that, in cases where specific tumor antigens are known to be expressed in a tumor, antigen presenting cells (for example, dendritic cells and macrophages) engineered to express a tumor-specific antigen, perhaps in conjunction with specific cytokines, may be even more effective vaccines. We are presently examining this possibility. Other gene therapy approaches for the treatment of cancer attempt to introduce tumor suppressor genes into tumor cells (*57*), to inhibit the expression of oncogenes in tumor cells (*58*), to render bone marrow cells resistant to the toxic effects of chemotherapy (*59*), and to introduce conditionally toxic genes into tumor cells (*60*).

A number of studies have focused on the development of gene therapies for acquired immunodeficiency syndrome (AIDS), cardiovascular diseases, and diseases of the central nervous system. Preclinical studies of potential gene therapies for AIDS have focused primarily on rendering cells resistant to human immunodeficiency virus (HIV) infection. A variety of intracellular vaccination strategies have been developed (*61*), directed toward either the inhibition of HIV infection or the inhibition of the release of virus progeny from cells (*62*). Targets of these therapies include T lymphocytes and hematopoietic stem cells. Cell-based vaccination strategies, such as those described above for cancer, and adoptive transfer strategies involving HIV antigen–specific T cells (*63*) are also being examined. In preclinical studies of potential gene therapies for cardiovascular disease, methods are being examined for genetically modifying cellular constituents of the vessel wall and the heart. Ex vivo approaches have included attempts to resurface specific arterial vessel segments with genetically modified endothelial cells or smooth muscle cells (*64*). The resurfacing of prosthetic vascular grafts with genetically modified cells has also been examined (*65*). In vivo therapies have involved the use of lipofection, retrovirally mediated gene transfer, adenovirus-mediated gene transfer, and injection of DNA to directly transduce cells of the vessel wall or of the heart (*66*). In addition, Rosenberg and co-workers have reported the use of continuous delivery of antisense oligonucleotides to regions of the vessel wall to inhibit intimal

thickening and restenosis (67). Preclinical models of therapies for diseases of CNS have focused on the development of methods for transplanting genetically modified cells into different regions of the brain (62).

Conclusion

The transplantation of transduced cells remains the most serious technical obstacle to the successful development of ex vivo gene therapies. Continued analysis of the specific features of existing methods of cell transplantation critical to their successful application in the clinic is vital. The emerging field of tissue engineering (40) may provide some new solutions to the transplantation problem. The major technical limitation of current methods for delivering genes in vivo is that the persistence of the transferred genes is transient, and therefore gene expression is transient as well. Although the transient expression of genes may prove to have clinical applications, particularly if gene transfer can be repeatedly performed, the applications are likely to be limited. Adenovirus-mediated gene transfer may provide more sustained gene expression than other methods of in vivo delivery and therefore is of considerable importance. This and other methods for in vivo delivery of genes should be more critically evaluated and compared.

Both the problems with cell transplantation and the transient nature of expression obtained with existing methods for in vivo delivery underscore the need to develop in vivo methods of gene transfer that lead to the stable transduction of cells. One possibility is that retrovirus vectors could be modified for use in in vivo gene delivery. Technically, this may require both the development of retroviral vectors that could integrate in quiescent cells and modification of the retrovirus particle itself to expand its host range and to improve its stability. The ability of HIV to integrate in some quiescent cells (69) may provide a strategy for achieving vector integration in quiescent cells (70), and the ability to incorporate chimeric envelope proteins or nonviral membrane proteins into retroviral particles may provide a means both for improving particle stability and expanding the host range. Modifications of the envelope protein may also permit the cell type–specific targeting of gene transfer obtained with receptor-mediated methods of gene transfer.

The development of receptor-mediated methods of gene transfer that result in the integration of DNA may also be feasible, either through the use of retroviral integration machinery or other well-characterized systems for integrative recombination, such as the *cre-lox* system (71). These latter systems are particularly interesting because they might provide a means of targeting integration to specific regions of chromosomal DNA and thereby reduce the risks associated with the random insertion of genes. In either case, the formidable technical challenge will be to assemble the necessary components into an appropriate complex, because in viral systems, the mechanisms for assembly and uncoating of viral particles represent distinct and very complicated biological processes (72). This convergence of the manipulation of viral and nonviral components in the development of gene transfer methods is likely to be a common theme of future research.

In addition to focusing on new methods of gene transfer, research will need to concentrate more on assessing the efficacy, rather than the technical feasibility, of potential gene therapies. Despite the potential application of gene therapies to a large number of inherited and acquired diseases, successful therapies will require more effective use of existing animal models and the development of creative, new models. Applications of gene transfer should also be considered more in the context of an overall clinical strategy for treatment, rather than as a distinct form of therapy. In the treatment of cancer, for example, it is likely that genetic manipulations of tumor cells, antigen-presenting cells, and lymphocytes will be most beneficial when used in conjunction with more conventional treatment (for example, systemic administration of cytokines, and chemotherapy).

The successful application of gene therapy will continue to require the efforts of investigators in the basic sciences, since basic science issues underlie many of the problems that need to be overcome in order for gene therapy to succeed. Many basic scientists and physicians remain skeptical of gene therapy because of the appearance that normal standards of scientific rigor have been suspended in the fervor to introduce gene therapy into the clinic. To attract such investigators, it will be critical to ensure that issues of scientific interpretation do not become confused with issues of clinical judgment and that scientific achievement is measured by peer review and consensus, rather than by the media.

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73. I thank T. Shenk, I. Verma, D. Housman, A. Berns, and members of my laboratory for helpful discussions.

New Challenges in Human in Vitro Fertilization

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This review assesses some scientific and ethical problems with human in vitro fertilization. Improved selection of viable embryos, better culture conditions, and greater understanding of the uterine environment will increase success and prevent multiple pregnancy. Further advances will also improve oocyte cryopreservation, in vitro maturation of oocytes, knowledge of sperm function, and sperm microinjection. Preimplantation diagnosis will help avoid genetic diseases and increase understanding of embryonic defects and the viability of zygotes. The greatest ethical problem with all these developments seems to be delivery of these complex treatments when health-care resources are increasingly limited.

Success Rates

Human in vitro fertilization (IVF) is surprisingly unsuccessful. In the United States, the overall birth rate per IVF treatment cycle is 14%, from 16,405 oocyte retrievals (*1*). In Britain, the live birth rate from each IVF treatment cycle started is 12.5% (*2*). Success is greater when more than one embryo is transferred simultaneously. Superovulation hopefully leads to fertilization of several oocytes, and it is common to transfer several embryos to the uterus, anticipating that at least one will implant.

Pregnancy resulted from 13% (184 out of 1436) of transfers when three or fewer embryos were transferred, 25% (238 out of 944) with four, and 26% (229 out of 871) with five or six embryos (*1*). On 154 occasions, seven or more embryos were transferred. Simultaneous transfer of multiple embryos increases the incidence of multiple pregnancy and the possibility of miscarriage and prematurity. Of triplets and quadruplets born after IVF, 64.1% and 75%, respectively, required admission to intensive care, often for weeks. Multiple pregnancy also has considerable social, economic, and psychological impact on parents. Prematurity after assisted conception was associated with a perinatal mortality rate of 27.2 per 1000 (*3*), three times the United Kingdom average for births after natural conception. The increased mortality was almost entirely due to multiple pregnancy. Consequently, it was informally agreed in Britain in 1987 that there should be limits to the number of embryos transferred at any one time, with a maximum of four embryos. In 1990, the British Government established the regulatory Human Fertilisation and Embryology Authority. Since then, most transfers have been restricted to no more than three embryos. For the last 3 years, we have seldom transferred more than two embryos simultaneously (*4*), and have been able to maintain pregnancy rates of 37 to 42% per transfer with only the occasional (<1%) triplet pregnancy.

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Can We Improve the Success Rate?

The quality of both the embryo and the uterine environment affects success. Individual human embryos only have a poor chance of development to fetal stages. After natural conception, possibly as many as 60% of very early pregnancies are lost (*5*). Most losses may be due to abnormalities of the conceptus; at least one-third of postimplantation embryos may be grossly abnormal (*6*). The situation is thought to be similar after IVF. At least 22% of eggs remaining unfertilized after insemination, and 16% of diploid two- to eight-cell embryos showed chromosomal abnormalities, particularly if some blastomeres were morphologically fragmented (*7*). As many as 30% of embryos may have genetic defects (*8*). A problem with all these studies is that karyotypic analysis of embryos is difficult; moreover, such studies have largely been done on poor quality embryos left over after transfer attempts. Women who become pregnant after embryo transfer tend to have more normal additional embryos (*9*). The spare embryos of women who miscarried tended to be chromosomally abnormal, as were 50% from women who developed ectopic pregnancy. The overall proportion of normal spare embryos was 13%, similar to the live birth rate per single embryo transferred (11%; 19 infants from 171 embryos). Thus, analysis of spare embryos might predict successful pregnancy.

Chromosomal aberration is more frequent when immature oocytes are fertilized. Superovulation decreases overall quality and maturity of oocytes and increases the number of adherent follicle cells (*10*). Superovulation preceded by desensitization of the pituitary by gonadotrophin-releasing hormone (GnRH) agonists, and reduction of luteinizing hormone concentration, before egg collection may improve egg maturation, which may in turn result in fewer miscarriages. GnRH antagonists and recombinant follicle-stimulating hormone (rFSH) may possibly help reduce the incidence of defective oocytes. However, we may have reached a biological limit in quality of embryos.

The goal for the future is the transfer of single embryos with a high chance of pregnancy (*11*). It is possible that the "best" embryos for transfer might be identifiable before transfer. Metabolic activity (*12*) or materials, such as cytokines, secreted into the culture media may be useful for predicting viability of embryos.

The success of embryo transfer after IVF decreases as the time after insemination increases. The reverse might be expected to be the case. Allowing IVF embryos to develop to the blastocyst stage should iden-