Articles

Progress Toward Human Gene Therapy

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Current therapies for most human genetic diseases are inadequate. In response to the need for effective treatments, modern molecular genetics is providing tools for an unprecedented new approach to disease treatment through an attack directly on mutant genes. Recent results with several target organs and gene transfer techniques have led to broad medical and scientific acceptance of the feasibility of this "gene therapy" concept for disorders of the bone marrow, liver, and central nervous system; some kinds of cancer; and deficiencies of circulating enzymes, hormones, and coagulation factors. The most well-developed models involve alteration of mutant target genes by gene transfer with recombinant pathogenic viruses in order to express new genetic information and to correct disease phenotypes—the conversion of the swords of pathology into the plowshares of therapy.

DURING THE PAST SEVERAL DECADES, THE PROMISING new concept of "gene therapy" has emerged, a concept based on the assumption that definitive treatment for genetic diseases should be possible by directing treatment to the site of the defect itself—the mutant gene—rather than to secondary or pleiotropic effects of mutant gene products. This involves an attack directly on mutant genes to replace or supplement defective genetic information with normal, functional genes. Gene therapy received much of its early theoretical support by the early 1970s (before the recombinant DNA era) from knowledge of the mechanisms of cell transformation by tumor viruses. Classes of DNA and RNA tumor viruses have evolved that carry out precisely those functions crucial to gene therapy, that is, the heritable and stable introduction of functional new genetic information into mammalian cells. Thus it was proposed that such viruses or other similar agents, deprived of their own deleterious functions, could be used as vehicles to introduce normal, functional genes into human cells to correct cellular defects and cure genetic disease (*1–7*).

At the outset, investigators studying genetic correction of human disease were faced with many serious conceptual, technical, and ethical problems, many of which surfaced after the studies by Cline *et al.* in 1980 on the use of cloned human β-globin genes to treat two patients with thalassemia (*8, 9*). Since that time, work with model systems has firmly established mutant genes as uniquely appropriate targets for therapy for at least some genetic disorders. Few discussions of gene therapy at scientific meetings and in publications still argue its need or potential place in medicine or its ethical acceptability, but rather they emphasize technical questions of efficiency of gene delivery and targeting and selection of suitable disease models. This review summarizes recent technical progress and discusses future clinical applications of methods of gene therapy.

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Strategies for Gene Therapy—Gene Replacement, Correction, or Augmentation

One form of gene therapy would involve specific removal from the genome of a mutant gene sequence and its replacement with a normal, functional gene. There is little or no conceptual groundwork to suggest how that might be accomplished. An ideal approach to gene therapy, gene correction, would entail specific correction of a mutant gene sequence without any additional changes in the target genome. Although gene correction has until recently seemed equally difficult, genomic targeting of foreign sequences (leading to specific gene sequence modification) has now been demonstrated in several mammalian systems. Several groups have shown that mammalian cells contain the enzymic and structural machinery for site-specific recombination of foreign DNA, in principle permitting targeted genetic modification of any known sequence (*10–13*). In most of these homologous recombination studies, targeted sequences have been introduced into cells by traditional gene transfer methods of calcium phosphate–mediated transfection, electroporation, or microinjection and have produced site-specific mutations in a number of loci, including the hypoxanthine-guanine phosphoribosyltransferase (HPRT) and the *int-2* loci in mouse embryonal stem cells. These methods will be applied to many other markers to study aspects of gene expression and to produce animal models for many human diseases (*14*).

More established than gene replacement or correction are several techniques of gene augmentation, that is, approaches for modifying the content or expression of mutant genes in defective cells by introducing foreign normal genetic sequences. During the past decade, a number of efficient methods have been developed to introduce functional new genes into mammalian cells (as discussed below). In many cases, it is possible to restore a genetic function by the addition of nontargeted but functional genetic information into nonspecific sites of the genome without the removal or correction of a resident, nonfunctional mutant gene. Inevitably, some insertional mutagenic events will result from the integration of foreign sequences at unusual sites in the genome and by the associated likelihood that gene expression from such ectopic transgenes is unlikely to be regulated faithfully. Nevertheless, because of the high efficiency of some kinds of vector-mediated gene delivery, this gene

augmentation model has received the most attention and has provided some of the most impressive progress in studies of gene therapy models.

Gene Transfer by Physical and Chemical Methods

Naked functional DNA can be taken up into mammalian cells by a number of physical methods, including coprecipitation with calcium phosphate, the use of polycations or lipids to complex with DNA, or by encapsidation of DNA into liposomes or erythrocyte ghosts (*15–18*). Exposure of cells to rapid pulses of high-voltage current (electroporation) (*19*) and even the introduction of DNA into cells by direct microinjection (*20*) or on high-velocity tungsten microprojectiles (*21*) also allows effective gene transfer and expression in target cells. A general characteristic of most of these transfection methods is the integration into the genome of multiple tandemly repeated copies of the transgene in a relatively stable form, although some investigators have found that integrated sequences can be highly unstable and can become inactive by a number of genetic and epigenetic mechanisms (*22*). The efficiency of physical transfection methods in vitro can approach or exceed 1% in suitable recipient cells.

Virus Vectors

The delivery of nucleic acids into mammalian cells can be made more efficient than permitted by the physical methods through the use of viral vectors capable of infecting virtually every cell in a target population (*23*). One can imagine many ways in which virus vector infection might damage host cells, including insertion of the vector into an essential gene of the host cell, the activation of a silent proto-oncogene by introduction of viral promoter or enhancer sequences, the activation of latent viruses encoded by the genome, or the rescue of infectious virus from the defective vector by recombination with cellular sequences. For the development of transducing viral vectors, most workers have concentrated on tumor viruses that integrate into host cell genomes and express foreign genes efficiently and stably without harming host cells. The first viruses used as expression vectors or gene transfer vectors for mammalian cells were transforming DNA viruses, including the papovaviruses (simian virus 40 and polyoma) and the adenoviruses (*23–26*). The genomes of these viruses are well understood and are still useful sources of transcriptional sequences for the construction of many current vectors, but because their capacity for foreign sequences is small, interest in them has waned as other potential vector systems have been developed. Obviously, such vectors will not be possible for the expression of many other disease-related functions because of the large size of many intact genes and the limitation of retroviral vectors to approximately 7 to 8 kb of added sequence.

Recently, the most useful and popular model vectors for the efficient introduction of foreign genes into target mammalian cells have been derived from murine and avian retroviruses. The mechanisms of infection, replication, integration, and gene expression from these replication-defective vectors have been reviewed elsewhere (*27–30*). Although retrovirus vectors are capable of infecting a broad class of cell types, cell replication and DNA synthesis are required for provirus integration; this effectively restricts efficient use of retrovirus vectors to replicating cells. To be useful clinically, retrovirus vectors would have to lead to efficient infection and stable gene expression. High-titer virus should be free of wild-type recombinants and, ideally, be capable of efficient and targeted delivery to specific sites in the genome and to appropriate cells and organs in vivo.

Expression and stability. Since the original reports of retrovirus-based vectors and helper cell methods, technical improvements have led to production of more useful vectors. Some of these contain only a single function expressed from the viral long terminal repeat (LTR). To simplify the isolation of suitable producer cells, other retroviral vectors also express a selectable marker, such as the transposon Tn5 phosphotransferase gene, to confer drug resistance to infected cells. In vectors with multiple transcription units, which express one function from the retroviral LTR promoter and another from an internal promoter, the relative position of the promoters expressing these two functions has a strong influence on the efficiency of gene expression and on virus production. Interactions between the promoters can result in marked reduction of the expression of one function as a result of selection for the other, and an upstream LTR promoter can interfere with expression from a downstream internal promoter (*31*). In a reverse orientation vector expressing the full genomic human β-globin gene under the control of its own promoter (*32*), globin gene transcription was efficient and specific in cells of the erythroid cell lineage.

A commonly cited advantage of retroviral vectors over other gene transfer tools is the presumed structural and functional stability of the integrated form of the retroviral vector or provirus. Although many retroviral sequences are stable, several studies have indicated that proviruses, like other transgenes, can show high-frequency structural and functional instability (*33, 34*) and that vector design, the nature of the target cell, the presence or absence of selection pressure, and the nature of the expressed genes can contribute to vector instability by mechanisms still not fully understood.

Retroviruses and their vectors are thought to integrate almost entirely into random sites in the cell genome, although some integration at specific preferred sites in the cell genome has been observed (*35*). Random integration will obviously lead to occasional insertional mutagenesis through the interruption of vital cellular genes or through the insertion of retroviral regulatory sequences that modulate the expression of flanking cellular genes. To avert problems of promoter interference and to reduce the likelihood of insertional mutagenic events, a number of investigators have designed retrovirus vectors that are devoid of their own promoter and enhancer sequences and are therefore transcriptionally disabled (*36, 37*). As expected, such disabled vectors have been shown to express vector-encoded genes exclusively from promoters introduced together with the gene, but so far their use has been limited by their low titers.

Vector titer and recombination. The preparation of high-titer retrovirus vectors has been aided by the development of efficient helper cell lines (*38, 39*) and the finding that the retroviral packaging signal required for encapsidation of virus RNA into particles includes a portion of the 5′ region of the *gag* gene in addition to the ψ site close to the 5′ LTR (*40*). Nevertheless, it has not been possible to reproducibly achieve retrovirus vector titers greater than approximately 10^6 infectious units per milliliter, whereas titers 10- to 100-fold higher would be necessary for many in vivo applications. Identification of more efficient helper lines and greater understanding of the mechanisms by which producer cells commit themselves either to express or to package provirus transcripts are needed before efficient vector delivery may be possible in vivo.

A continuing problem with the use of defective retrovirus vectors has been the eventual appearance of wild-type virus in producer cells, presumably through recombination between the transfected vector plasmid and endogenous retrovirus-like sequences. To reduce the opportunities for recombination, several investigators have produced helper cell lines that express the *gag*, *pol*, and *env* genes

from separate integrated plasmids (*41, 42*). Virus titers obtained with these helpers have been reported to be as high as with the more conventional single-plasmid helper lines.

Vector targeting. Although highly efficient gene transfer is already a feature of retroviral vectors, it has not been possible to incorporate site specificity of integration. One preliminary but promising approach has been the recent report of restoration of function to a defective *neo* gene that was integrated in rat cells by infection of the cells with an integration-defective retrovirus vector containing a partially deleted, nonfunctional *neo* gene (*43*). The appearance of G418-resistant cells was attributed to homologous recombination between the two different deleted *neo* genes. It remains to be seen whether efficient site-specific integration is compatible with mechanisms of retroviral integration or whether site-specific targeting will require other kinds of vector delivery.

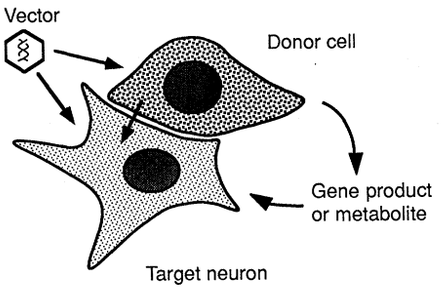

Fig. 1. Genetic correction of CNS defects. Phenotypic modification of CNS functions can be produced by implantation of donor cells previously modified genetically in vitro. The new function can then be supplied by cell-cell contact or by transport or diffusion to target CNS cells. Alternatively, the gene transfer vector can be introduced into CNS either directly or through the circulation.

Other Virus Vectors

Retrovirus vectors are useful for many kinds of in vitro gene transfer studies, but the problem of relatively low titers limits their use for some in vitro and most in vivo studies. Expression vectors derived from viruses such as vaccinia virus (*44, 45*), adeno-associated virus (AAV) (*46, 47*), herpesviruses, bovine papilloma virus (*48*), and others offer features that may make them attractive for some applications of long-term genetic modification of mammalian cells. Human AAV viruses have a number of potential advantages over retrovirus vectors, including the fact that they are ubiquitous in humans and can be concentrated to titers exceeding 10^9 infectious units per milliliter. They are completely nonpathogenic integrating viruses that require coinfection with helper adenoviruses or herpesviruses for replication of their 5-kb single-stranded DNA genomes (*49*). A prototype transducing AAV vector has been shown to express an integrated *neo* gene stably in human and mouse cells in vitro (*46*). As is true of other systems (including, to some extent, the retrovirus vectors), there continue to be difficulties with the AAV vector system, including the presence of contaminating cytopathic or pathogenic helper viruses, relatively uncharacterized mechanisms of integration, and a paucity of information on the long-term consequences of integration and gene expression from the integrated AAV provirus.

Because of the restriction of retrovirus vectors to replicating cells and the need for high-titer vectors for transduction and expression of foreign sequences in nonreplicating or fully differentiated postmitotic cells such as neurons, hepatocytes, and other retrovirus-refractory cells, interest is increasing in vectors derived from several classes of nonintegrating viruses, such as the herpesviruses. Portions of their large genomes (150 kb) (*50*) may be expendable for virus replication and other viral life cycle functions, and they could therefore have much higher capacities for added foreign sequences than most other known vectors; they may also be able to transfer and express large, intact genes. Natural herpes infections often become latent in neural cells, implying that herpes-based vectors that mimic latent infection have the potential for long-term gene expression in neural cells.

Expression vectors developed from herpes saimiri (*51*), herpes simplex virus type-1 (HSV-1) (*52*), cytomegalovirus (*53*), Epstein-Barr virus (*54*), and pseudorabies viruses (*55*) have been shown to express reporter genes in mammalian cells in vitro. One group has recently used recombination to prepare a vector expressing the human HPRT cDNA and has demonstrated expression of the human gene in HPRT-deficient rat neuroma cells in vitro (*56*). Since the recombinant vector in this case was replication competent, transgene expression was limited to short periods of time after infection. Noncytopathic herpes-based vectors can be prepared by several methods, including the use of plasmids containing replication and packaging signals linked to the gene to be expressed, or the introduction of foreign sequences into replication-defective virus mutants by recombination. In either case, preparation of the vectors requires the use of (i) either temperature-sensitive or other conditional virus mutants to provide replication and encapsidation functions or (ii) nonconditional modified helper viruses and appropriate cell lines that complement the defects of the helper virus. To generate replication-defective vectors that can be maintained free of wild-type virus for prolonged periods of time, other workers have prepared a plasmid-based HSV-1 vector able to express an *Escherichia coli* β-galactosidase gene in cultured peripheral and sensory neurons (*57*). There is little information available on the long-term fate or stability of gene expression from these vectors, on the development of latency in infected cells, on the rescue of latent herpesvirus by exposure to vectors, or other effects of the vector on cells. Nevertheless, these preliminary studies should lead to considerable interest in development and further characterization of herpes-based vector systems.

In Vivo Delivery and Disease Models

Bone marrow. Much of the work in developing techniques for clinically applicable gene transfer has centered around complementation of genetic defects in target cells in vitro followed by the implantation of the resulting genetically modified cells into a suitable organ in vivo (Fig. 1). The target organ for implantation must be the site of relevant disease, easily accessible, manipulable in vitro, susceptible to genetic modification methods, and, ideally, should contain either nonreplicating cells or cycling stem cells to perpetuate a genetic correction. If only differentiated, replicating cells are infected, the newly introduced gene function will be lost as those cells mature and die. Finally, it should be possible to reimplant genetically modified cells into the organism in a functional and stable form. Because the mammalian bone marrow satisfies all of these criteria, it has been one of the most attractive organs for early studies of gene therapy.

Certain cells of the mammalian bone marrow are susceptible to infection with retrovirus vectors, and a number of studies in mice have shown that retrovirally delivered transgenes in infected bone marrow cells in vitro can, under some conditions, restore enzymic activities for months in vivo after repopulation of the bone marrow space (*58–61*). Evidence that authentic stem cells have been infected with the vectors has come from experiments in which transplanted marrow cells expressing a retrovirally transduced *neo* gene have been removed from a primary recipient animal and transplanted to

secondary recipient animals. Because the bone marrows of some secondary recipient animals were also repopulated by neomycin-resistant cells, the presumption was that only authentic, cycling totipotential stem cells rather than more differentiated progenitor cells could have been responsible (*59*). Direct demonstration of retrovirus vector infection of authentic totipotential stem cells has not been reported. Rather than stable long-term expression, the common experience in many laboratories has been that expression from retrovirally transduced genes in mouse bone marrow is transient and unstable. The limited experience with larger animals such as dogs and monkeys has so far been equally disappointing (*62–64*). Unstable provirus gene expression may result from (i) genetic or epigenetic structural provirus instability, (ii) the possibility that only committed precursor cells and not truly totipotential stem cells can be infected by or can express retrovirally transduced genes, or (iii) the possibility that the repopulated marrow expresses cell lineages cyclically from different precursor cells, some expressing a transgene efficiently, whereas others do not.

Retroviral gene expression is known to be poor in some highly undifferentiated cells such as embryonal carcinoma (EC) cells and possibly bone marrow stem cells because of an inability of the viral LTR enhancer to function in such cells (*65*). However, plasmids containing enhancers isolated from genes that are normally expressed in EC cells, from the rare EC cell successfully infected with a retrovirus vector, or from an infectious myeloproliferative syndrome virus did allow efficient transient gene expression in EC cells (*66–68*). Similarly, plasmids containing a reporter gene together with an enhancer sequence isolated from polyoma mutants able to infect EC cells were able to express the gene transiently after transfection into EC cells (*67–69*). Gene expression from retroviral or other vectors containing these EC-permissive sequences has not yet been reported.

On the basis of these animal studies, a genetic approach to the treatment of human bone marrow diseases has been proposed. The most suitable and heavily studied current models are those of the immunodeficiency diseases caused by defects of adenosine deaminase (ADA) and purine-nucleoside phosphorylase (*70, 71*), chronic granulomatous diseases (*72*), and Gaucher's disease (*73*). These and similar disorders are currently treated either symptomatically or, in the case of ADA deficiency, by enzyme replacement therapy or by bone marrow transplantation from histocompatible donors. The recent use of purified ADA stabilized by polyethylene glycol (*74*) may make effective therapy accessible to more ADA-deficient patients without resorting to gene therapy. Similar advances in traditional therapy have not occurred in Gaucher's disease or chronic granulomatous disease.

Among the earliest theoretical targets for gene therapy were erythroid cell disorders of hemoglobin expression, including sickle cell anemia and the thalassemias. However, none of the early gene transfer techniques were capable of bringing globin transgenes under faithful gene regulation, and unbalanced expression of the several globin genes is in itself deleterious. Therefore, it has seemed until recently that these diseases might not be ideal as models for studies of gene therapy. However, the correction of the mouse thalassemia in transgenic mice with vectors that retain regulatory genomic sequences (*75*) and the recent discovery of regulatory sequences far upstream from the human β-globin cluster (*32, 76*) suggest that appropriately regulated and efficient expression of globin genes may indeed become feasible. In the case of sickle cell anemia, the increased intracellular viscosity responsible for erythrocyte trapping and destruction results more from the presence of the insoluble sickle β-globin chains than from the mere absence of normal β-globin chains; therefore simple restoration of normal β-globin synthesis without a shutdown of S-globin synthesis is unlikely to have any beneficial therapeutic effect (*77*).

Liver. The liver plays a central role in human metabolism and in the expression of many genetic diseases. The adult mammalian liver contains relatively quiescent, differentiated hepatocytes that are refractory to retrovirus infection. However, differentiated rat hepatocytes are susceptible to retrovirus vector infection during a dedifferentiation phase of growth in vitro (*78*). Investigators have taken advantage of this finding to develop retrovirus-mediated methods to express a number of genes in primary hepatocyte cultures in vitro, including the disease-related genes for the human receptor for low-density lipoproteins (LDLR) (*79, 80*), phenylalanine hydroxylase (*81*), and α_1-antitrypsin (*82*). The α_1-antitrypsin promoter has been incorporated into a phenylalanine hydroxylase retrovirus vector to confer liver specificity to retrovirus gene transfer (*83*). The tasks now are to develop efficient means of reimplanting genetically modified hepatocytes into recipient animals to restore a genetic defect and, ideally, to develop vectors that can be introduced directly into hepatocytes in vivo without resorting to in vitro genetic manipulation and cell reimplantation. Long-term survival of hepatocytes implanted into rat peritoneum and spleen has been reported (*84, 85*), suggesting that implantation of genetically modified hepatocytes to produce a phenotypic change in vivo should be feasible. On the other hand, the awkwardness of isolating a large number of hepatocytes, presumably by liver biopsy, from a patient for in vitro genetic manipulation before autologous transplanation makes direct vector delivery into the liver highly desirable.

Central nervous system (CNS)—genetic and nongenetic disease. The neurologically devastating Lesch-Nyhan disease, resulting from defects in the X-linked HPRT gene, has been one of the most useful models for development of general techniques for human gene therapy and of approaches to the genetic modification of CNS functions (*86–89*). Experience with this disorder and others such as Alzheimer's and Parkinson's diseases has made it clear that genetic approaches to the dysfunctioning mammalian CNS are not entirely straightforward. Useful models for genetic approaches to therapy of CNS disorders are difficult to identify for the following reasons: (i) little is known about normal or abnormal CNS function, (ii) the organ and many of its cells are inaccessible both physically and physiologically, and (iii) most disorders affecting CNS function are likely to be multigenic and multifactorial. Furthermore, most of the presumed target cells for CNS disorders, neurons, are postmitotic and therefore refractory to infection with retroviral vectors. My colleagues and I have proposed a combination of in vitro gene transfer and cell grafting into specific regions of the mammalian brain as an approach to the restoration of function in at least some CNS defects (*90*) (Fig. 1). It seems preferable for both technical and ethical reasons to design an autologous cell genetically modified to produce the desired product rather than to rely on naturally occurring human donor cells. In a test of that hypothesis, it was recently reported that rat fibroblasts expressing a retrovirally transduced mouse nerve growth factor (NGF) cDNA and implanted into a lesioned rat brain protected cholinergic neurons from degeneration and death after injury of the fimbria fornix (*91*). The relevance of this NGF study to any specific human disease remains to be established, although the protected neurons are analogous to those that are correlated with the memory deficit in human patients with Alzheimer's disease. Studies are now under way in many centers to examine the potential role of NGF in the pathogenesis or treatment of Alzheimer's disease (*92*). Similar approaches to the delivery of useful agents in models of Parkinson's disease are also under way. If such combined gene transfer–implantation approaches were proved effective, they would establish the feasibility for delivering therapeutic gene products or metabolites through grafting of genetically modified cells in the treatment of genetic, developmental, degenera-

tive, infectious, or traumatic kinds of dysfunction of the mammalian CNS. This approach is less likely to be effective for CNS disorders that reflect more global defects of neuron function, as in Tay-Sachs and other lysosomal storage diseases, some neurotransmitter disorders, possibly the Lesch-Nyhan syndrome, and many other disorders. For at least some of those defects, it will probably be important to develop neuron-specific gene transfer techniques, including possibly herpes and other "neurotropic" vectors to deliver and express new genetic information in neurons distributed diffusely in CNS.

Cancer. Because it is likely that most human cancer is caused by, or is associated with, aberrant gene expression, human cancer should be considered a genetic disease (*93*). Recent characterization of the role of growth factors and proto-oncogenes in development and cell proliferation has begun to suggest genetic approaches to augment the current chemotherapeutic or irradiation treatments. At the moment, the simplest genetic models involve neoplasia resulting from deficiencies of cancer suppressor genes such as those apparently associated with the development of the human cancers retinoblastoma (Rb) and Wilms' tumor. These cancers presumably arise from the inactivation of both alleles of a wild-type gene, therefore the cancer phenotype might be suppressed and possibly even reversed by restoring functional expression from a wild-type gene. My colleagues and I have provided support for this approach in a study showing that infection of Rb and osteosarcoma cells with a retrovirus vector expressing the wild-type Rb gene led to morphological changes and to a reversion of their anchorage-independent growth properties in vitro and, in the case of the Rb cells, to the suppression of their tumorigenic properties in nude mice (*94*). Further studies are required to characterize the long-term effects of the restoration of suppressor gene expression on cell properties and the replication and tumorigenicity of cells homozygous or heterozygous for Rb defects and to determine whether it is possible to induce reversion of existing tumors. Similar studies with other suppressor gene defects will be important to establish this general approach to therapy.

Inactivation of dominantly acting oncogenes is conceptually more difficult but is suggested by new approaches to site-specific targeted mutagenesis. Several methods are now available for the expression of toxin genes in cells (*95, 96*). In principle this would permit the targeted destruction of tumor cells if unerringly specific delivery can be accomplished reproducibly. In another approach, targeted introduction of a drug sensitivity gene specifically into tumor cells is designed to make them uniquely susceptible to pharmacological treatment, as in the introduction of the HPRT gene into thioguanine-resistant human leukemia cells to make them sensitive to purine analog antimetabolites (*97*).

The potential for modulating the expression of oncogenes and other genes through the use of genetic information in the form of antisense oligonucleotides suggests another approach to the suppression of the cancer phenotype (*98–102*). Although the mechanisms involved in inactivation of gene expression by antisense sequences are not thoroughly understood, it is probably necessary to deliver high concentrations of antisense information to compete with either efficient gene translation or possibly with the function of transcription factors. Chemical modifications of naked oligonucleotides have increased their cellular uptake and stability (*103*), and considerable efforts are being made to develop more efficient targeted and vector-mediated methods for introduction of the sequences into cells and into whole animals.

Other target organs and disease models. Therapeutically useful products, including hormones, serum proteins, other humoral or diffusible proteins, and even some low molecular weight metabolic products, may be produced in a whole animal by cells other than the normal sources. If suitable gene regulation can be ensured, it should be entirely irrelevant to an organism whether a humoral factor is supplied from its usual cell of origin or from a genetically modified ectopic cell. Skin cells such as fibroblasts and keratinocytes are especially attractive because they are readily available, can be grown in vitro from affected patients, are easily transformed genetically by vectors, and can be reintroduced readily by autografting into donor animals. This in vitro gene transfer and autograft concept has served as the theoretical basis for genetic therapy of the hemophilias and other disorders of the serum proteins, insulin-deficiency forms of diabetes mellitus and other hormone defects, α_1-antitrypsin deficiency, and other enzyme- or gene product–deficiency diseases (*104, 105*). When such cells are implanted into animals, the newly synthesized product secreted into the circulation may thereby correct a deficiency and a disease phenotype of a distant cell or organ. Studies in mice have shown effective production and secretion into the circulation of functional factor IX for several weeks, at least until the recipient animals mounted an immune response to the foreign protein (*106*). Implantation into immunologically unresponsive animals or the use of species-specific genes are likely to avert adverse immune responses.

Infectious diseases. Eventual genetic approaches to the treatment of disease are obviously not limited to genetic diseases. Infectious diseases, including acquired immunodeficiency syndrome, result from the action of the infectious agent's genes, and an understanding of those genes and their functions will suggest suitable targets for therapy. Many investigators are studying the feasibility of cell-specific delivery of antisense sequences, toxin genes, or other genes into cells to interfere with expression of the pathogenic genetic functions.

Direct Vector Delivery in Vivo

In contrast to in vitro gene transfer followed by cell implantation, a theoretically more attractive but less well developed approach would involve direct introduction of a gene transfer vector into a target organ in vivo. This approach would require the preparation of concentrated or very high titer preparations of the transducing viral vector or other physical gene transfer vehicles. Retroviruses may not lend themselves easily to in vivo delivery because virus titers are generally quite low compared with the number of target cells that must be genetically modified and because they are generally not cell tropic.

Other physical agents including naked plasmids or cloned genes encapsidated in targetable liposomes or in erythrocyte ghosts have been used to introduce genes, proteins, toxins, and other agents directly into whole animals. In vivo liposome-mediated gene delivery has led to expression of foreign insulin I and preproinsulin genes in recipient rats (*107, 108*), and several studies have demonstrated that direct injection of naked calcium phosphate–precipitated plasmid into rat liver and spleen or of protein-coated plasmid into the portal vein resulted in gene expression in the liver (*109*). These studies have suggested that, under some circumstances, direct organ-specific gene transfer by physical means may be effective. To improve the efficiency of vector delivery, it may be possible to take advantage of tissue or organ tropisms, for example the use of vectors derived from the neurotropic viruses such as rabies or herpesviruses, for gene transfer into CNS. Because most pathogenic animal viruses are not truly tissue tropic for infection but express their cytopathic functions preferentially in one tissue or another, it is more likely that tissue-specific vectors will be produced through the use of these rather more promiscuous vectors containing promoter, enhancer, and other sequences to confer tissue specificity to gene expression.

Replication-competent, infectious but nonpathogenic forms of vectors will probably also find eventual use for gene delivery in vivo in cases when delivery to a single organ may not be sufficient and when body-wide gene expression would be necessary.

The Problem of Faithful Gene Regulation

No matter how efficient and stable the techniques of gene delivery and expression may become, most potential clinical applications are likely to require faithful regulation of the foreign gene expression. Too much, too little, and inappropriately timed gene expression will make disease correction difficult. Although great strides have been made recently in dissecting regions of genes responsible for regulating the levels of gene product, there is still much to be learned about the many additional levels of transcriptional and translational control exerted on genes before medically relevant genetic complementation can become commonplace.

Ethical Considerations and Human Experiments

As is true with all other new procedures in medicine, therapeutic studies of gene therapy in human patients will be performed with imperfect knowledge when technical uncertainties and imperfections are outweighed by clinical needs (*110–112*). The balance between uncertain harm and desired benefits has been examined carefully by a number of religious, ethical, and public policy bodies, including Pope John Paul II in an address to the 35th General Assembly of the World Medical Association, the World Council of Churches, the Parliamentary Assembly of the Council of Europe, the Presidential Commission for the Study of Ethical Problems in Medicine and Biomedical Research, the Office of Technology Assessment, the National Council of Churches, the medical research councils of Canada and Australia, the governments of Denmark and the Federal Republic of Germany, and others. All have agreed that somatic genetic manipulation for the purpose of ameliorating disease should be pursued (*113*). Other observers have disagreed (*114*).

The long-awaited first applications of gene transfer techniques to human disease were expected to involve a therapeutic restoration of a disease-related enzyme activity. Approval has recently been given by the NIH Recombinant Advisory Committee, by the director of NIH, and by the Food and Drug Administration for a study of the fate of tumor-infiltrating lymphocytes (TIL cells) (*115, 116*) after in vitro infection with a retrovirus expressing *neo* (as a selectable marker) and subsequent reimplantation into donor cancer patients. Information from this study is intended to guide further studies on the implantation of vector-infected cells, but the results from this study are likely to be highly specific for the TIL cells, the vector, and the transgene. Similar studies should eventually be carried out for other combinations of genes, target cells, and vectors before large-scale human clinical studies can be undertaken.

Deliberate or inadvertent modification of human germ line cells can presumably occur by many of the same methods described here. The potential role of germ line manipulation for the prevention of genetic disorders is far less clear than is somatic cell modification, and one response to the possibility of germ line genetic modification has been to suggest that it is so full of technical and ethical uncertainties that it should not be performed. However, it seems unwise and premature to take such a severe position, and it has been suggested that the need for efficient disease control or the need to prevent damage early in development or in inaccessible cells may eventually justify germ line therapy. This most problematical of all issues in gene therapy requires much more examination.

Summary

The development of viral and other methods to transfer functional genes stably into human and other mammalian cells continues to make progress, and a logically consistent technical and ethical basis has been established for the application of these techniques to the amelioration of human disease. Efficient gene transfer in vitro and in vivo and genetic correction by site-specific targeting of many disease-related genes are becoming feasible. Many problems remain to be solved, and continuing responsible and informed scientific and public discussion is required to identify suitable applications. Answers to the commonly asked technical questions, "When can we expect to see the first successful human clinical applications?" and "Which disease is the best candidate?" are of minor importance compared with the realization that this conceptually new approach to the treatment of disease is a response to a medical need and is achieving increasing medical, scientific, and ethical acceptance.

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Genetic Engineering of Livestock

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Genetic engineering of livestock is expected to have a major effect on the agricultural industry. However, accurate assessment of the consequences of transgene expression is impossible without multigenerational studies. A systematic study of the beneficial and adverse consequences of long-term elevations in the plasma levels of bovine growth hormone (bGH) was conducted on two lines of transgenic pigs. Two successive generations of pigs expressing the bGH gene showed significant improvements in both daily weight gain and feed efficiency and exhibited changes in carcass composition that included a marked reduction in subcutaneous fat. However, long-term elevation of bGH was generally detrimental to health: the pigs had a high incidence of gastric ulcers, arthritis, cardiomegaly, dermatitis, and renal disease. The ability to produce pigs exhibiting only the beneficial, growth-promoting effects of growth hormone by a transgenic approach may require better control of transgene expression, a different genetic background, or a modified husbandry regimen.

THE ABILITY TO INTRODUCE NEW GENES INTO THE GERM line of an animal and thereby produce proteins outside their normal environment and separated from their usual physiological control mechanism has been extremely valuable for studying numerous aspects of gene expression as well as other questions in biology (*1*). Most investigations of gene expression in animals have utilized the mouse as an experimental animal. However, gene transfer has recently been extended to domestic animals (Table 1).