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The Biotechnology of Gene Therapy

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ABSTRACT

The prospect for correcting highly morbid or fatal inherited diseases, or ameliorating cancer and acquired, deadly infectious diseases such as AIDS using gene therapy is very exciting. Numerous recent advances in molecular biology make it possible, not only to identify and locate genes associated with human inherited disorders and cancers, but to potentially correct these disorders with functional genes. These advances include more rapid gene identification, isolation and sequencing techniques, a better understanding of the functions and relationships between genes and their products in vivo, the development and study of human and model organism genomes, elucidation of genetic disease pathology using animal genetic disease models, advanced computer amino acid and nucleotide sequencing software and data bases, and the development and use of novel chemical, physical, and viral vector gene delivery methods. Functional genes are introduced using two approaches, ex vivo and in vivo gene therapy. In ex vivo therapy, autologous cells are removed from the patient, genetically altered by inserting the functional gene, characterized, and then returned to the patient; in in vivo therapy, functional genes are packaged for delivery directly into the patient, where cellular uptake and gene expression occurs. Scores of clinical trials have been federally approved to treat patients with a variety of inherited disorders, cancers, and acquired diseases using these two approaches. Roadblocks to long-lasting gene therapy include understanding more completely the biological functions of somatic cells or organs targeted for gene therapy, targeting appropriate host cells and achieving high gene delivery rates in these cells, regulating and sustaining gene expression through optimal DNA insertion into chromosomes such that other cellular functions are not adversely affected, and the prevention of vector-induced diseases or cancers. Ethical considerations regarding proper use of somatic gene therapy and the potential for germline gene therapy must also be seriously considered. The prospect of permanent correction of highly morbid or fatal maladies using gene therapy could prove to be one of the great advances in public health and could revolutionize the

identification and gene–drug treatment of a broad spectrum of inherited and acquired human diseases.

INTRODUCTION

Over 4000 human inherited diseases are associated with either dysfunctional, nonfunctional, or missing gene-coded proteins (1). Observed differences in the structure and function of defective proteins may be caused by single-point gene mutations, by multiple, discrete mutations, or by deletions of large segments of critically important genomic DNA (2). In addition to hereditary diseases, a wide variety of other maladies, such as AIDS, arthritis, atherosclerosis and heart disease, and cancer can be traced back to impaired function of a specific gene or genes important in the immune system or in metabolic regulatory systems. In the past, geneticists traced inherited disorders by first identifying and analyzing defective structural proteins, and then tracing back to find the responsible structural gene. Geneology of families with hereditary diseases, as well as the use of murine genetic disease models, when available, assisted researchers in understanding disease pathology and identifying the chromosomal location of defective genes. Historic examples include familial hypercholesterolemia, sickle cell anemia, and blood diseases caused by the thalassemia syndromes (1).

In the last decade, the identification, location and sequencing of defective genes have been facilitated through numerous advances in biotechnological methods and techniques. These include the use of highly specific restriction enzymes and bacterial plasmid vectors, hybridization assays, gene cloning, polymerase chain reaction (PCR) amplification, chromosome mapping, chromosome walking, and restriction fragment length polymorphisms (RFLP) (2–6). Through the research efforts of thousands of academic, government, and private sector researchers worldwide involved in the Human Genome Initiative, over 1700 human genes have been identified and mapped, laying the groundwork for developing gene therapy, a spectrum of corrective gene–drug treatment regimens for inherited, acquired, and infectious diseases.

GENE THERAPY

Gene therapy entails complex molecular methods for inserting functional (good) genes into mammalian cells which have defective (bad) genes in order to ameliorate or cure specific diseases (7–9). Thus, inherited acute diseases and late-onset neuromuscular diseases caused by defective genes which direct the synthesis of poorly functional or nonfunctional proteins, or which fail to produce proteins at all, can be prevented or ameliorated by providing the patient's cells with working copies of the desired gene. Functional genes are introduced using two approaches: ex vivo and in vivo gene therapy. In ex vivo gene therapy, target cells are removed from the patient, genetically altered with the functional gene, characterized, and then returned to the patient by injection or infusion. In contrast, in vivo gene therapy does not require the removal of patient cells. Functional genes are prepackaged for injection or aerosol delivery directly into the patient, where cellular uptake of the gene complex occurs, followed by expression in vivo.

Obviously, gene therapy has great potential for reducing morbidity and mortality not only in inherited monogenic diseases but also in malignancies and transmissible diseases, such as AIDS (9) and Epstein–Barr virus (EBV) lymphoproliferative disease, through therapeutic gene delivery into target cells or immune restoration (10). Since 1990, over 130 somatic cell gene therapy trials have been approved by the National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC) for inherited diseases, numerous cancers, cardiovascular diseases, and viral diseases. Enrollment in these trials is over 600 patients (11). Although the numbers of patients treated in earlier trials was small, enrollment in ongoing and future trials should increase to more satisfactory levels as additional patients and physicians learn of specific gene therapy trials and more trials receive RAC approval. Preparation of functional genes, delivery methods, candidate diseases, cost considerations, manufacturing and quality control, and social and ethical issues for gene therapy are outlined below.

Gene Delivery Methods

To date a number of methods have been developed for insertion of functional foreign genes into cells. In general, they may be divided into two categories, transfection and transduction (Table 1).

Transfection

Both chemical and physical techniques have been developed to facilitate insertion of genes into eukaryotic and prokaryotic cells. These include chemical methods

Table 1

Methods for Functional Gene Insertion into Mammalian Cells

Transfection
Chemical
Calcium phosphate coprecipitation
Lipofection (synthetic anionic and cationic liposomes)
Receptor-mediated gene delivery (soluble DNA/glycoprotein–polycation conjugates)
Physical
Naked DNA plasmid injection
Electroporation
Bioballistic (particle acceleration)
Viral transduction: recombinant viruses
Adenovirus (AV)
Adeno-associated virus (AAV)
Bovine papillomavirus (BPV)
Herpes simplex virus (HSV)
Simian virus 40 (SV40)
Vaccinia virus
Mouse mammary tumor virus (MMTV)
Molony murine leukemia virus (MoMLV)

using calcium phosphate coprecipitates, synthetic liposomes, and bifunctional polycation conjugates. Calcium phosphate coprecipitation is very simple to perform and effective for delivering genes to a wide variety of cell types, but it is highly variable and can be cytotoxic (12). The technique must be standardized for each cell type transfected. Anionic (pH-sensitive) liposomes carry genetic material within their aqueous interiors, but because both the liposome and functional gene are highly negatively charged, only small amounts of DNA are enclosed in the liposomes. The alternative use of cationic liposomes was shown recently to offer multiple advantages over traditional anionic liposomes, such as increased shelf life, higher transfection efficiency, and the ability to complex larger portions of genomic DNA than retroviral vectors (13). A synthetic, cationic liposome containing 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) has been shown to transfer a foreign gene for cystic fibrosis into mammalian cells both in vitro and in vivo using a murine model system (14). DOTAP was also shown to be an effective vector for transfection of breast cancer cell lines (15). Both anionic and cationic synthetic liposomes offer good to excellent insert capacity, the ability to enter large numbers of target cells, in vivo delivery, and transfection of quiescent (nondividing) cells (16).

A novel transfection method using polycation molecular conjugates has been demonstrated to effect delivery of foreign genes into mammalian cells both in vitro and in vivo (17). Termed "receptor-mediated gene delivery," bifunctional glycoprotein–polycation conjugates are complexed to plasmids containing the desired gene and appropriate promoters. The resulting soluble DNA/glycoprotein–polycation amine complex is recognized by cell surface receptors on the target cells and endocytosed. Receptor-mediated gene delivery is a highly efficient method of delivering large copy numbers of foreign DNA (up to 10^3 copies/cell). Thus, by carefully designing the glycoprotein–polycation conjugate, exquisite cell targeting of large amounts of foreign DNA becomes possible. It is important to note that during manufacture, soluble DNA plasmids must be mixed with the correct proportions of conjugate to assure that the foreign gene is optimally delivered and expressed, and that the DNA conjugate remains as intact plasmids in target cells for only a limited period of time (about 14 days). This most likely is due to the fact that few DNA conjugates escape from the endosome before lysosomal fusion and digestion occurs, limiting its utility as a vector method. The inability to escape lysosomal degradation might be overcome by complexing the DNA conjugate with adenovirus, which also infects host cells through receptor-mediated endocytosis but has an endosome escape mechanism. This could prevent lysosomal digestion and allow the DNA conjugate complex to reach the nucleus.

Physical methods of gene transfection include naked DNA plasmid injection, electroporation, and bioballistic or particle acceleration gene delivery. Direct injection of DNA plasmids is appealing due to its simplicity (18), but it does not result in appreciable percentages of transfected target cells (1–3%) and works for the most part only in heart and skeletal muscle (19). Electroporation, the formation of transient hydrophilic pores in cell membranes by an electrical field (20), is a popular method for delivering DNA into both eukaryotic and prokaryotic cells (21,22). Commercial, portable electroporators are available which precisely control voltage and other parameters important in electroporating all cell types, maximizing gene transfection efficiency, and minimizing host cell trauma. Bioballistic gene delivery, which was originally developed to facilitate the injection of transgenes into plant cells to create genetically engineering food crops, has been used to transfect mammalian cells (23). In this method, microparticles coated with genetic material are forcefully injected into cells, delivering the functional gene; the method is fast, simple to perform, and versatile (24). Bioballistic gene delivery can be used in both ex vivo and in vivo delivery of genetic material, and skin is often the target tissue for

in vivo delivery of functional genes to laboratory animals. However, transfection of internal organs requires surgery.

A major advantage of physicochemical and synthetic gene transfection methods is that they are less risky than viral vector gene transduction. In general, they also offer lower toxicity, protect genetic material from lysosomal degradation, do not initiate a host immune response, can be targeted to specific host cells or tissues, do not integrate into the host genome, give high (but short-lived) gene expression, and should be able to be produced commercially in large amounts in accordance with precisely defined manufacturing and quality control specifications at a reasonable cost. For these reasons, they have been used mainly for in vivo gene therapy (8). A major disadvantage of these methods is that transfected genes are only expressed for a short period of time soon after gene delivery, as integration into the host cell genome does not occur efficiently. Newer versions of liposomes, containing retroviral reverse transcriptases or retroviral integrases, may facilitate functional gene integration and long-lived gene expression. Presently, transfection methods account only for 5% of all clinical trials being performed (25).

Transduction Using Recombinant Vectors

A number of viral-based vectors for inserting functional genes into mammalian cells in vitro have been described in the scientific literature. These include adenovirus (AV), adeno-associated virus (AAV), herpes simplex virus (HSV), Moloney murine leukemia virus (MoMLV), mouse mammary tumor virus (MMTV), simian virus 40 (SV40) and vaccinia virus (see Table 1). Retrovirus vectors are presently the most widely used for transduction of mammalian cells in clinical trials (25) because they allow functional genes to be integrated in a single chromosomal region, assuring long-term expression. However, presently all viral vectors are technically deficient in at least one of the following ways: (*a*) suboptimal vector viral gene transduction, (*b*) difficult to manipulate in the laboratory, (*c*) limited ability to carry large segments of foreign DNA, (*d*) host immune response to the vector with repeated treatments, (*e*) toxicity, (*f*) limited host range, (*g*) inability to transduce nondividing cells, and most importantly, (*h*) diminished gene expression over time. In addition, each of these vectors must first be made incapable of reproducing itself as an infectious agent in the host cell. This is termed making the virus "replication defective." It is accomplished by deleting critical nucleic acid sequences from the vector's structural genes in such a way that it cannot replicate in host cells. These replication-defective vectors can still be manufactured in vitro in sufficient quantities using specially developed "packaging cell" lines (26,27).

Adenoviruses (AV), which are the second most popular gene therapy vector after retroviruses, have important positive and negative attributes. They infect nondividing cells easily, their genetic material does not integrate into the host genome (remains extrachromosomal), they are very stable, and are readily manufactured in large quantities (8). However, AV vectors can only hold about the same amount of genetic material as retroviral vectors (about 5–8 kilobases), they are immunogenic, and can infect germline cells as well as somatic cells if used for in vivo gene therapy. Importantly, AV vectors are more likely to revert to competent infectious virus particles in the host than replication-defective retroviral vectors (19). In order to prevent reversion, replication-defective AV vectors were developed with missing segments of E1 genes. These vectors can still be produced in adequate numbers in cell lines genetically engineered to express the E1 genes (28). Adeno-associated viruses (AAV) are also becoming more popular as vectors. Although AAV integrate into the host cell genome less efficiently than retroviruses and can hold only about half as much genetic material as adenoviruses and murine retroviruses (about 4.5 kilobases), they have a broad host range, are nonpathogenic, and allow site-specific integration in chromosome 19 of the human genome (8,13).

Vaccinia viruses have been intensely studied and engineered over the past 10 years for use as vectors and as live vaccines against a variety of other infectious agents, including hepatitis B virus (29), HSV (30), influenza virus (31,32), and malaria parasites (33). Recombinant vaccinia preparations retain infectivity, are thermally stable, have a broad host range, and can hold at least 25,000 base pairs of foreign DNA (34). However, the naked vaccinia DNA is noninfectious and because its genome is so large, vaccinia vectors are difficult to engineer in the laboratory. Standard restriction endonuclease cleavage, followed by insertion of a foreign gene and transfection into suitable host cells to produce recombinants, are not feasible and must be replaced by marker rescue methods (35). In addition, foreign gene expression can only occur by ligating the foreign gene sequence downstream of a translocated vaccinia promoter, as vaccinia DNA-dependent RNA polymerase does not recognize nonpoxvirus promoters (36). Many other viral vectors do not have this promoter requirement.

Despite the limitations noted above, retroviruses have retained popularity as therapeutic vectors because they have been optimized genetically through evolution to efficiently insert, integrate, activate, and express their own genetic material in mammalian target cells with close to 100% efficiency (8). The majority of federally approved gene therapy trials utilize retroviral vectors (25). The challenge has been to capitalize on innate retroviral transduction abilities without compromising the health and welfare of the patient. Using murine cells during initial retroviral vector production is potentially hazardous, as endogenous, competent murine retroviruses in the cell cultures may interact with defective retroviral vectors, producing replication-competent vectors (25). Alternative methods have been described to decrease the risk of producing such competent vectors (37). Even though numerous replication defective retroviral vectors have been produced through sequence deletions of the *env, gag,* and *pol* genes (38), virulent wild-type retrovirus production from packaging cell lines has been documented (39), and several primates in one study developed lymphomas after being treated with bone marrow cells transduced using retroviral vector preparations found to contain replication-competent retroviruses.

Safe retroviral design and production is only one aspect of the gene therapy strategy. The desired human gene for insertion must be identified, isolated, cloned, and characterized using a variety of molecular biological methods (40). It is then engineering with appropriate promoters and enhancers into what is termed a "cassette." This functional gene "cassette" must then be encoded into the retroviral vector RNA in a way that the gene and its promoters retain their sequence integrity yet do not significantly reduce or prevent the vector's capacity to enter the host cell, reverse transcribe, integrate, and express the functional gene. Taken all together, viral vector engineering is very tedious, time consuming, and expensive, and will likely present scale-up and manufacturing problems and quality control issues in the future (14).

The optimal vector will have the following attributes: (*a*) exquisite tropism for the target cell (selective cellular uptake), (*b*) the ability to transduce nondividing as well as dividing cells, (*c*) the ability to carry a large functional gene "payload," (*d*) selective integration within the genome, (*e*) transcription stabilizers, (*f*) gene expression modulators, (*g*) minimal cytotoxicity, (*h*) cell compartment tags, and (*i*) the ability to be manufactured and quality controlled at a reasonable price (19,25,41). Present vectors have some but not all these favorable attributes. In the future, synthetic vectors composed of genetic material from a variety of nonretroviral and retroviral sources may provide optimal gene payload, safe and effective delivery, and optimally functional, long-lived integrated genes with minimal production and quality control costs (19,42,43).

Murine Genetic Disease Models

Laboratory animal models have been essential in understanding the genetics, affected metabolic regulatory systems, pathologies, and treatments of inherited diseases. Murine genetic disease models are very useful molecular genetics research tools and will prove invaluable in establishing and validating gene therapy strategies for human inherited diseases. Unlike nonhuman primates, mice are less expensive, small in size and easier to house and maintain, and have rapid gestation and generation times (20 and 60 days, respectively) (44).

A number of murine genetic models for human diseases have been described in the literature, and some of these are listed in Table 2. Under ideal circumstances, the murine model displays all the pathological manifestations of the human disease. Historically, murine genetic disease models were accidentally discovered in mouse colonies. More recently, murine disease models have been produced through specifically induced mutagenesis. Highly mutagenic compounds, such as *N*-ethyl-*N*-nitrosourea (ENU) have been used to produce germline lesions of a single base pair in mice, which is very useful for human monogenetic disease modeling. However, ENU-induced genetic lesions are random, and up to 1000 offspring of affected mice must be screened to find the desired gene mutation (44). Another mu-

Table 2

Murine Genetic Disease Models with Applications for Gene Therapy

Deficiency	Disease Model
Apolipoprotein E	Atherosclerosis and hypercholesterolemia
Cystic fibrosis transmembrane conductance protein	Cystic fibrosis
Dystrophin	Muscular dystrophy
Glucocerebrosidase	Gaucher's disease
Glucose-6-phosphate dehydrogenase (G6PD)	G6PD deficiency syndrome
Hemoglobin, α-chain	α-Thalassemia
Hemoglobin, β-chain	β-Thalassemia
p53 mutation	Spontaneous tumors

tagenesis method, termed "targeted gene replacement," uses embryonic stem cells and is capable of producing genetic lesions in predetermined loci of chimeric mice. Embryonic stem cells are mutated in vitro in tissue culture, then introduced into mouse blastocysts to colonize all tissues, including the germline. The method has been refined to allow a single mutation introduction into selected genes using homologous recombination, assuring a monogenic disease model for gene therapy experimentation.

DISEASES TARGETED FOR GENE THERAPY

Many inherited, monogenic diseases, cancers, cardiovascular diseases, and virus-induced diseases such as AIDS have been selected for human clinical trials worldwide, using a variety of novel target cells, gene insertion methods, and activation strategies. Selection has been based mainly upon factors such as accumulated knowledge of the locations and sequences of the defective genes, the functions of protein products coded for by the defective genes, adequate murine or simian disease models, the onset of pathology of the diseases, the functions and sequences of viral pathogen genes, and the availability of affected individuals. Table 3 lists the genetic diseases, carcinomas, and acquired diseases which have been approved thus far for gene therapy trials in the United States. Scores of protocols have received National Institutes of Health RAC approval over the past 5 years and many more are awaiting approval, but the number of enrolled patients is still quite small (45). Gene therapy trials are ongoing in other countries, such as France (46) and Italy (47). Diseases and disorders for which gene therapy could prove effective are described in the text below.

Inherited Diseases

The first gene therapy experiment approved and conducted by the NIH occurred in September 1990 (9). A 4-year-old girl with severe combined immunodeficiency (SCID), caused by a defect in the gene coding for the enzyme adenosine deaminase (ADA), was chosen for treatment. The production of defective ADA results in accumulation of toxic by-products in the blood which eventually kill immune lymphocytes, leading to a wide variety of respiratory infections caused by otherwise nonpathogenic microbes, and often early death. Functional genes were introduced using the ex vivo approach. Autologous T lymphocytes were first removed from the girl, placed in cell culture and transduced with recombinant plasmid vectors containing functional ADA genes ex vivo, and then infused back into the child on four occasions over a 4-month period. The child showed significant improvement and over the past 4 years has led a relatively normal life, assisted by occasional booster infusions of ADA gene-transduced lymphocytes and continued (precautionary) administration of the polymerized version of the enzyme, polyethylene glycol ADA (PEG-ADA) (9). Subsequently, two other successful gene therapy studies for treatment of ADA-induced

Table 3

Diseases for Which Clinical Trials of Gene Therapy Are Ongoing

Inherited Disorders	Cancer	Infectious Diseases
Alpha-1 antitrypsin deficiency	Brain	AIDS (HIV)
Chronic granulomatous disease	Breast	
Cystic fibrosis	Colon	
Familial hypercholestemia	Leukemia	
Fanconi's anemia	Liver	
Gaucher's disease	Lung	
Hemophilia	Lymphoma	
Hunter's syndrome	Melanoma	
Peripheral vascular disease	Mesothelioma	
Purine nucleoside phosphorylase deficiency	Multiple Myeloma	
Rheumatoid arthritis	Neuroblastoma	
SCID (ADA)	Ovarian	
	Prostate	
	Renal cell	

SCID have been reported, each involving two patients in the United States and in Italy (47). In the Italian study, researchers transduced both bone marrow cells and peripheral cells with copies of a functional ADA gene using a genome-integrating retroviral vector. They reported that after treatment termination, the transduced bone marrow cells eventually replaced the peripheral cells with ADA-producing red cells, granulocytes, and lymphocytes. This suggests that gene therapy successfully integrated ADA gene copies into long-lived progenitor cells, abrogating the need for continual follow-up treatments.

Encouraging results have also been observed with cystic fibrosis (CF), a highly morbid, hereditary disease which afflicts about 30,000 people in the United States. It is the most prevalent disease among Whites. Death frequently occurs at a young age, often before the age of 30 (38). After the isolation and characterization of the defective gene on chromosome 7 in 1988, it was learned that the defective proteins interfered with channeling of sodium and chloride ions in and out of cell membranes (48). Subsequent in vitro and in vivo gene therapy studies using vaccinia, retrovirus, and adenovirus vectors demonstrated the ability to successfully transduce epithelial cells and in some cases to transiently correct the ion transport defect (49,50). A recent CF gene therapy safety trial using a first-generation AV vector was halted due to excessive pulmonary cytotoxicity; a second-generation AV vector which demonstrated less toxicity in a primate model will be substituted and the trial restarted (13). Other gene delivery systems being used in clinical trials include AAV vectors and synthetic liposomes (19).

Inherited diseases and their target cells which will utilize retroviral vector-mediated gene delivery in NIH RAC-approved trials include: (*a*) chronic granulomatous disease (myeloid cells), (*b*) familial hypercholesterolemia (hepatocytes), (*c*) Fanconi's anemia (hematopoietic progenitor cells), (*d*) Gaucher's disease (peripheral blood cells and stem cells), and (*e*) Hunter's syndrome (lymphocytes). Besides a CF clinical trial, an alpha-1 antitrypsin deficiency human trial was approved using liposomes as transfection vectors.

About 1% of neurological diseases are caused by inherited gene defects, affecting tens of thousands of people worldwide. Interestingly, the incidences of inherited neurological diseases—such as Alzheimer's disease, Gaucher's disease, Hurler's disease, Parkinson's disease, and Tay–Sachs disease—are at least as high or higher than presently targeted nonneurologic diseases, such as CF, familial hypercholesterolemia, and SCID (42). As the majority of inherited neurological diseases are monogenic (i.e., each disease produces a single defective enzyme or transcription regulator), and affected individuals are for the most part free of disease from birth through adulthood, people with these types of inherited diseases are potential candidates for presymptomatic genetic testing and follow-up gene therapy. For example, early diagnosis of neurodegenerative disorders such as Parkinson's disease might allow enough time to conduct novel therapies before irreversible brain damage occurs.

Gene therapy could also replace very expensive periodic protein replacement therapies, such as Ceredase®, currently used to treat Gaucher's disease, and recombinant Factor VIII (Kogenate™) for hemophelia A. The therapeutic objectives with inherited neurodegenerative diseases would be to insert functional genes into target brain cells that would produce sufficient quantities of specific enzymes so that the disease processes would be reversed or ongoing neurodegeneration arrested. Targeting such a large group of people has the potential of significantly reducing morbidity and mortality worldwide, increasing human productivity, and significantly reducing health care costs associated with traditional care. However, ultimate success of gene therapy in this group of diseases will depend upon the number of factors, including patient age, clinical history (to include other debilitating diseases or concurrent infections), and stage of progression of the targeted disease (42).

There are presently three methods of delivering genes into brain tissue: fetal cell transplantation, bone marrow transplantation, and vector transduction. However, fetal cells are difficult to acquire due to ethical considerations, and bone marrow transplantation, although demonstrated to effect enzyme transfer to host brain cells (51), may not enjoy wide use, as this form of treatment suffers from inadequate numbers of histocompatible donors. Gene delivery through vector transduction may prove the best strategy for neurodegenerative diseases.

Both in vitro and in vivo transduction model systems have been developed showing the utility of DNA and RNA viral vectors to transfer foreign genes into neurons (52), but long-term gene expression in these cells has to date been low, due to retroviral vector-induced toxicity. The use of nonretroviral vectors may prove less toxic than retroviral vectors, especially where gene expression over long periods of time is required. Neurological disease treatment using ex vivo transfection or viral transduction of autologous bone marrow cells might also prove a good approach.

Cancer

Research indicates that the development of cancers is monoclonal in nature, that is, derived from a single aberrent cell which undergoes a pathologic alteration in one or more of its genes, rendering it "immortal." Whereas the strategy of inherited monogenic disease gene therapy is to replace poorly functioning or nonfunctioning genes with functional ones, this would probably not work well for treating cancer, as it would require transduction of 100% of the tumor cells in the body. A more appropriate strategy for cancers might be to emphasize gene-induced destruction of rapidly reproducing cancer cells, especially with those tumors which are inoperable (42). To this end a number of strategies have been used to fight a variety of cancers.

The first gene therapy treatment for cancer was an NIH RAC-approved two-patient clinical study on advanced melanoma that utilized autologous, genetically engineered lymphocytes. Tumor infiltrating lymphocytes (TILs) from malignant moles were harvested and transduced ex vivo with retroviral vectors genetically engineered with copies of the gene coding for tumor necrosis factor (TNF). The TNF engineered TILs were stimulated to reproduce in vitro by incubation with the cytokine interleukin-2, then reinfused into the patients over a period of time, and TIL-mediated tumor regression was followed (38).

The fact that cancer cells divide rapidly is also being used in the gene therapy strategy for certain cancers. Brain tumor cells could be transduced with the HSV-1 thymidine kinase gene, as the retroviral vectors transduce dividing tumor cells and not quiescent neurons. The transduced cancer cells would then express large amounts of viral thymidine kinase and become susceptible to the toxic effects of the neucleoside drug ganciclovir. Selective drug activation of 5-fluorocytosine has also been investigated similarly in cancer, and other drug activation systems based on galactosidase, glucuronidase, and nitroreductase may hold promise. Nucleoside drug-induced toxicity may then be limited to replicating cancer cells, sparing normal tissue (53). While the great majority of gene therapy trials for cancer and inherited diseases use retroviral vectors (19), the use of nonretroviral vectors, such as AV, HSV-1, or parvovirus may be useful also in treating cancers, by reducing toxicity. Thus, the insertion of cytotoxic genes into cancer cells may prove an effective, cancer cell specific treatment for a variety of solid tumors.

Another gene therapy approach to cancer involves vaccination. In order to elicit a stronger immune response to tumor cells, vaccines composed of tumor cells genetically altered with genes coding for cytokines have been developed (15). Although such studies to date have used genes coding for interleukin-2, other cytokines, such as interleukin-4 or TNF, may also prove useful. In any event, presymptomatic diagnosis of cancer followed by treatment with protective genes before oncogenesis and/or metastasis might prevent disease or significantly reduce morbidity and mortality.

AIDS

The utility of gene therapy to treat infectious viral diseases caused by viruses such as HIV, the cause of AIDS, lies in the premise that viral DNA expression in the host cell can be halted. Effective treatment of HIV infection to date has been hampered by the facts that the virus: (a) infects several cell types, including T-helper lymphocytes; (b) continuously replicates during the early phase of infection; (c) is capable of becoming resistant to present drug treatments, such as AZT; and (d) progressively decays the host's immune system, leading to death (54–56). Gene therapy, nucleic acid based therapeutic vaccines, and immune reconstitution are currently being evaluated as potential treatments for HIV infection (57). Gene therapy strategies include prevention of HIV replication through: (*a*) inactivation of HIV regulatory proteins by therapeutic gene-expressed RNA decoys (RNA-based suppression) (58), or through ribozyme-induced cleavage and inactivation of HIV RNA (59,60), (*b*) suppression of viral regulatory functions, such as protein synthesis, by therapeutic gene-produced mutant essential proteins which compete with normal HIV proteins for viral RNA transport from the nucleus to the cytoplasm (61), and (*c*) production of inducible toxins or conditional toxins through "suicide gene" insertion that kill only HIV-infected cells (62,63).

The nucleic acid based therapeutic vaccine strategy entails direct injection into the muscle of naked DNA plasmids or retroviral vectors containing the HIV gene of interest. Induced cells express the HIV gene product (antigen) and the injected person then mounts an immune response to HIV-infected cells and free virions. Both humoral and cellular immune responses have been shown to occur in mouse and nonhuman primate models after the direct injection of HIV genes, due to de novo protein synthesis by gene-altered target cells (64,65).

Immune restoration entails the acquisition, in vitro expansion and reinjection of cells such as cytotoxic T lymphocytes and/or $CD4^+$ lymphocytes into patients

(66). These cells types are acquired, transduced, and expanded ex vivo with antiviral genes, cytokine genes, or marker genes (for in vivo tracking), and then reinfused into patients. A number of HIV gene therapy clinical trials have been approved by the NIH RAC. Gene products delivered into patient lymphocytes include HIV-cleaving ribozyme genes, a transdominant *Rev* protein-based suppressor gene, and a thymidine kinase suicide gene/hygromycin B marker gene (19,57). Although numerous studies have demonstrated suppressed viral activity in vitro using gene therapy, the lack of small animal models has hampered research and development efforts. Advanced mouse models using genetically engineered stem cells and functional human T lymphocytes are being developed and could greatly enhance HIV research efforts.

If gene therapy is to prove successful in the treatment of AIDS, it should be initiated a short time after HIV infection. This would reduce the damage caused by replicating virus upon lymphoid organs (57). In addition, AIDS gene therapy should be designed so as not to be circumvented by genetic variation of the HIV virion (67). Due to the complexity of AIDS and the involvement of a large number of cell types, gene therapy in conjunction with antivirals and immune restoration may prove to be the best approach.

Vascular Cell Proliferation

Gene therapy may also find utility in treating cardiovascular diseases (68). The ability to apply in vivo gene transfer to atherosclerotic arteries in a rabbit model (69), the demonstration of gene transfer-induced vascular cell hyperplasia and angiogenesis (70), and other related studies, led to approval by the NIH RAC of human gene therapy for cardiovascular diseases in late 1994 (71). Gene delivery systems include catheter administration of protein conjugates, liposomes, and viral vectors, but viral vectors are preferred as they are more efficient in therapeutic gene insertion (68). Vascular smooth cell proliferation has been implicated both in human atherosclerosis and in restenosis of coronary arteries after balloon angioplasty. Arterial restenosis occurs in about 30–50% of patients and is a very expensive complication of this surgical procedure (68). Recently, gene therapy was used as a treatment for balloon-induced arterial restenosis in rat and porcine models systems (72). Carotid arteries in rats and femoral arteries in pigs were injured by balloon angioplasty. Retinoblastoma-gene-product (Rb) was inserted in an adenovirus vector and delivered at the injured sites to transduce local intima cells. The Rb vector codes for a nonphosphorylatable form of the human Rb. In the normal cell, when human Rb is phosphorylated, it activates transcription factors that induce vascular cell proliferation. Therefore, cells which are transduced with the therapeutic Rb gene are rendered unable to progress from the resting phase (G0) to the active phase (G1/S), where vascular cell proliferation occurs. After human Rb gene treatment, migration of smooth muscle cells into the injured sites was significantly reduced in both the rat and pig models. Interestingly, use of the Rb vector in rats and pigs did not induce an inflammatory response or other toxic effects. However, AV vector transduction efficiency of intima cells is lower in the presence of naturally atherosclerotic lesions than in normal arteries (73), so the clinical utility of gene therapy in naturally damaged arteries needs further clarification. A human trial for peripheral artery disease using a plasmid-containing tumor angiogenesis factor (TAF) gene delivery system has been approved by the NIH RAC (19). Localized endothelial cells will be treated in vivo using a TAF plasmid-coated catheter. Thus, patients who have cardiovascular diseases may ultimately benefit from localized in vivo gene therapy of vascular tissues.

BIOTECHNOLOGY FIRMS IN GENE THERAPY

The potential of gene therapy to revolutionize medical treatment of inherited diseases, cancer, and infectious diseases has led to the establishment of for-profit gene therapy biotechnology firms. Although the first gene therapy trials were performed by researchers at the National Institutes of Health in Bethesda, Maryland (9), numerous subsequent NIH RAC-approved clinical trials are ongoing at many other medical centers, often in collaboration with private gene therapy companies.

Table 4 lists biotechnology and health care companies with interests in gene therapy. Some of these firms were venture capitalist funded spin-offs from university- or medical-center-based genetics research programs; others were existing biotechnology companies that initiated gene therapy research projects as logical extensions of in-house genetic research and development programs (38). Many gene therapy firms have ties to the NIH, universities, and/or medical centers, and cooperate with government and academic medical researchers and geneticists to develop gene therapy methods and model systems for specific diseases. In addition, large health care firms, such as Rhone-Poulenc-Rorer, Inc., and

Table 4

Biotechnology and Health Care Companies in Gene Therapy

Company	Location	Comments
Aastrom Biosciences, Inc.	Ann Arbor, MI	Stem cell gene therapy for cancer
Alexion Pharmaceuticals, Inc.	New Haven, CT	Retroviral gene therapy
Avigen, Inc.	Alameda, CA	Gene therapy vectors
Cell Genesys, Inc.	Foster City, CA	T-cell gene therapy for AIDS, cancer and genetically engineered universal donor cells
Chiron Corp.	Emeryville, CA	Recombinant IL-2 gene therapy
Genetic Therapy, Inc.[a]	Gaithersburg, MD	Cancer, anemia, and AIDS gene therapy
Genvec, Inc.	Rockvill, MD	Cystic fibrosis gene therapy
Genzyme Corp.	Cambridge, MA	Gaucher's disease gene therapy
Immune Response Corp.	Carlsbad, CA	Colon cancer gene therapy
Molecular Therapies	Gaithersburg, MD	Various gene therapies
Rhone-Poulenc-Rorer, Inc.	Collegeville, PA	Investments in gene therapy firms
Rgene Therapeutics, Inc.	The Woodlands, TX	Ovarian cancer gene therapy
Sandoz, Ltd.	Basel, Switzerland	Investments in gene therapy firms, including Genetic Therapy, Inc. and purchase of Systemix, Inc.
Somatix Therapy Corp.	Alameda, CA	Cancer, hemophilia, neural drugs
Systemix, Inc.[a]	Palo Alto, CA	Bone marrow gene therapy for HIV
Targeted Genetics Corp.	Seattle, WA	Cystic fibrosis/cardiovascular and Gaucher's disease gene therapy
Viagene, Inc.	San Diego, CA	Interferon-gamma therapeutic vector and hepatitis C gene therapy
Vical, Inc.	San Diego, CA	Cancer and infectious disease gene therapy

[a]Acquired by Sandoz Pharma, Ltd.

Sandoz Pharma, Ltd., have invested heavily in small gene therapy firms, essentially as development partners. One in particular, Sandoz Pharma, has actually purchased a small biotechnology firm, Genetic Therapies, Inc., to assist in gene therapy drug development. Genetic Therapies has obtained exclusive worldwide rights to a patent application detailing the use of a herpes simplex-tk/ganciclovir gene approach to destroy brain tumor cells. The patent application was sublicensed to Genetic Therapies by Bristol-Myers Squibb, which originally licensed the patent application from Massachusetts General Hospital (74). Other large firms with interests in gene therapy include Baxter Healthcare, Bayer AG, Bristol-Myers Squibb, Chugai Pharmaceuticals, Hoffmann-LaRoche, Merck, Pasteur Merieux, and Schering Plough. Obviously, the potential for tremendous profits exists for biotechnology and pharmaceutical companies that develop safe and effective gene therapy methods which could augment or supercede conventional drug therapies.

ETHICAL CONSIDERATIONS

Gene therapy is fraught with scientific, social, legal, ethical, and financial problems, and these have been discussed in numerous articles (75–78). Will the introduced genes function as well as their normal counterparts, and what adverse side affects will be associated with gene therapy treatments? For example, in human clinical trials for treatment of SCID, periodic booster infusions of transduced lymphocytes and continued administration of the polymerized version of the enzyme (PEG-ADA) have been performed to prevent recurrence of the disease (45), and a cystic fibrosis trial was halted due to vector-induced toxicity (13). Important adverse side effects could also result from poor integration or from multiple gene integrations within the same target cell. The inserted functional gene might integrate in an area of a chromosome which prevents the expression of another essential gene product, resulting in disrupted cell development and/or cell function. Thus, if enough tar-

get cells are affected, gene therapy could itself result in a completely different monogenic disorder. Even worse, functional gene integration might accidentally activate oncogenes or inactivate tumor suppressor genes, causing cancer (19). The possibility that replication-defective vector preparations accidentally contaminated during manufacture with replication-competent vectors might cause cancer in patients undergoing gene therapy can also not be excluded.

Germline Gene Therapy

To date, no germline gene trials have been approved by the NIH RAC, and it is unlikely to do so for the foreseable future (79). However, research continues in this area (80). There are important technical and ethical problems regarding germline therapy. First, the numbers of patients enrolled in current gene therapy trials is small, so that there is still not enough data regarding safe and efficacious gene therapy of somatic cells. Second, once germline gene therapy has been well defined experimentally and shown to be safe and efficacious, there is great potential for abuse. The implementation of eugenics by national decree, as in Nazi Germany over 50 years ago, is a chilling possibility, particularly in totalitarian regimes (38). To this end at least one researcher has returned federal funds for cancer research, to protest possible germline engineering misuses of his research (79). It is also possible that patients or parents of patients might want cosmetic germline gene therapy for short stature, or eye or hair color. However, there are also positive aspects to germline gene therapy. Gene therapy of germline cells such as sperm and eggs could prevent the development of inherited diseases not only in the affected individuals but in their offspring, abrogating the need for further somatic cell gene therapy every generation.

Cost Considerations

Currently, gene therapy is very expensive (9,38). Genetically engineering functional genes and vectors with desirable characteristics, the development and optimization of delivery methods, and long-term medical support requires talented, dedicated researchers and physicians, expensive laboratory equipment, and computer hardware and software, and is extremely time consuming. The high cost of gene therapy trials is even more striking considering that only small numbers of patients are currently enrolled, each receiving individualized treatment (45). Besides the basic science involved, the feasibility of gene therapy will be determined by a number of financial factors, such as the ability to diagnose diseases early enough to allow cost-effective treatment, and the cost-effectiveness of gene therapy for patients with certain types of inherited diseases or cancers (42). Indeed, very basic ethical questions regarding the worth of cures for certain highly morbid or deadly genetic or acquired diseases have not yet been addressed (38). The Cano Commission, chaired by Dr. Jean Paul Cano, has predicted that by the year 2010, nearly 30% of health care costs in Western nations will arise from advances in genetics-related diagnostics and therapies (46). This amounts to about US$300 billion on a worldwide basis. If many thousands of patients are to be treated successfully over th next 20-year period, then gene therapy procedures will have to become less expensive and less complicated. In this regard, eventual application of germline gene therapy could significantly reduce the cost of repetitive gene treatments every generation in afflicted families. In the near term, however, scale-up and manufacture of gene therapy "drugs" which are highly pure and consistent from lot to lot will be necessary, and will also be expensive and highly controlled, as are other drugs for human use.

Early Diagnosis of Inherited Diseases

The patient benefits and cost-effectiveness of optimized gene therapy regimens will have to weighed against traditional diagnosis and drug treatment strategies in the next 10 years, especially if gene therapy becomes a widely applicable corrective therapy over a broad range of inherited and acquired diseases (38,46). It is quite possible that certain inherited diseases and cancers will be easier to diagnose early and will be treated with greater success than others, justifying the expense of these treatments over others. Profits, which have driven the development of many therapeutic drugs, will most likely be directly related to the prevalence of the diseases in the population, the ease of diagnosis and gene treatment, and the financial benefits of gene therapy intervention over expensive traditional long-term medical treatments (reducing the type and number of drug treatments, hospital stays, laboratory tests, and follow-up treatments) (38). Examples of inherited diseases presently requiring expensive, long-term treatment regimens are Gaucher's disease, hemophilia, and famil-

ial hypercholesterolemia. If gene therapy proves efficacious yet very expensive, it is possible that only the wealthy would be able to afford such specialized treatments.

CONCLUSIONS

Somatic cell gene therapy is still in its infancy but holds great promise as an alternative treatment strategy for correcting inherited monogenic diseases, cancer, and infectious diseases. Ex vivo gene therapy has now shown promise for a few diseases, such as SCID, and in vivo gene therapy is being evaluated for cardiovascular diseases. It is not yet known whether somatic cell gene therapy will be a medically plausible remedy for the many different inherited diseases, cancers, and acquired diseases afflicting humans. The success of gene therapy may vary with each disease and may depend upon factors such as the type of gene insertion strategy (transfection versus transduction), the ability of the gene to integrate and express itself adequately in the target cell, and the life span of the targt cell in vivo. In order to assure optimal insertion, stable integration, and adequate function of genetic material, the gene delivery system must have multiple desirable properties. These include: (a) safety, (b) efficient gene delivery and extended (long-term) expression, and (c) lack of toxicity. Synthetic vectors with desired attributes from many viral and nonviral sources could be developed to optimize gene delivery. As gene therapy methods and delivery systems are improved, as more genetic disease models are developed, and as more genes are mapped and sequenced through the Human Genome Project and retroviral pathogen research, effective and long-lasting gene therapy scenarios for many disease states may become a reality. Gene therapy could eliminate the need for long-term parenteral administration of traditional drugs and problems associated with medication compliance. However, costs involved in the manufacturing and qualitry control of gene-drugs and in the delivery of functional genes will be important factors and may prevent the development of gene therapies for certain diseases. Finally, gene therapy for the unborn fetus and eventually even germline gene therapy may become plausible ways of permanently correcting gene defects and preventing their transmission to offspring.

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