Human Gene Therapy

What is gene therapy?

Simply stated, the basic principle underlying gene therapy entails detecting a genetic defect associated with a disease and correcting that defect by administering the correct DNA sequence to the defective cells. Initially, this principle was applied in cases where providing an additional normal copy of a defective gene aimed to restore the synthesis of a missing protein, such as an enzyme. The concept of gene therapy has since been broadened to include many more procedures, of which the most important are:

1) The gene transferred may code for a protein that is not necessarily missing but that may be of therapeutic benefit and difficult to administer exogenously. IL-2 or antitumor cytokines are two examples. This form of gene therapy aims to enhance in vivo production of potentially therapeutic proteins.

2) Another form of gene therapy involved transducing non-physiological sequences, which have antiviral activity, such as antisense oligonucleotides or sequences.

3) Finally, so-called suicide genes can be transduced into undesirable cells (cancer cells or infected cells) to sensitize them to specific substances. When these substances are administered subsequently, they will trigger selective destruction of the targeted cells.

Somatic and germ line gene therapy

If the target cell for transduction is a sex cell (sperm, egg, or their precursors), then the genomic modifications will be passed on to the individual's descendants (germ line gene therapy). If the target cell is a somatic cell (somatic gene therapy), then the genomic modification will not be hereditary.

Somatic gene therapy consists of introducing a gene into the somatic cells of an individual with the expectation that the gene will be expressed and that its product will exert a therapeutic effect. This is the form of gene therapy that is being developed in humans and also widely performed in other animal species. In theory, somatic gene therapy guarantees the integrity of the genome of germ line cells and avoids the potential risk of propagating an artificial transgene within a species.

Germ line gene therapy means modifying the genome of the sex cells of an individual or of the fertilized ovum (zygote). In both cases the genetic modification is present in the individual's gametes and will be transmitted to the offspring to become a hereditary trait of the species. To date, such manipulations have never been authorized in humans, and remain confined to laboratory animals. Germline gene therapy is not illegal in the USA, though no trials have ever been performed or submitted for approval. It remains illegal in Europe.

A. Techniques of Gene Therapy

These are summarized as follows:

Gene Transfer
Chemical: calcium phosphate transfection
Physical:
- electroporation
- gene gun
- microinjection
- Ultrasound-mediated transfection
Recombinant virus vectors: transduction
- retroviral vectors: Moloney murine leukemia virus, HIV virus
- adenovirus
- adeno-associated virus
- herpes simplex virus
-vaccinia virus
-baculovirus
-poxvirus
-hybrid viruses (not yet used in humans)
-other viruses

Non-viral vectors for gene therapy
- Liposomes
- Ligand-polysylne-DNA complexes
- Dendrimers
- Receptor-mediated endocytosis
- Synthetic peptide complexes
- Artificial viral vectors
- Artificial chromosomes

Gene Administration
Implantation of genetically engineered cells
Direct injection of naked DNA or genes: systemic or at target site
Receptor-mediated endocytosis

Gene regulation
Regulation of expression of delivered genes in target cells by Locus Control Region (LCR) technology
Molecular switch to control expression of genes in vivo
Promoter element-triggered gene therapy

Repair of defective genes
This involves correction of the gene in situ

Gene replacement
Excision or replacement of the defective gene by a normal gene

Inhibition of gene expression
Antisense oligodeoxynucleotides (ODN)
Ribozymes

Moreover, gene therapy can be also subcategorized as ex vivo and in vivo:
Ex vivo gene transfer techniques usually involve the genetic alterations of cells (cell lines or human cells), mostly by use of viral vectors, prior to implanting these into the tissues of the living body.
In vivo gene therapy means direct introduction of genetic material into the human body. In vivo gene delivery may be local (in situ) or systemic. In situ gene therapy means the introduction of genetic material directly into a localized area in the human body, e.g. within the tumor.

B. Gene Repair and Replacement

The ideal treatment of a genetic disease would be total replacement of the defective gene with a normal copy of the gene. This is difficult to achieve in practice. The technique of homologous recombination involves switching a section of defective gene with donor DNA containing the normal nucleotide sequence but the efficiency of this technique is poor. The inserted gene must be in the proper location for regulation of gene expression and reconstitution of the disturbed function.
Site-specific homologous recombination can be achieved more efficiently by the use of oligonucleotides that bind to the mutation and a DNA-damaging agent damages the mutated nucleotide. This activates the normal repair mechanisms to correct the defect and restore the proper sequence. This concept forms the basis for the antisense technology, as we shall see later.

One technique of gene repair is known as “chimeraplasty” which aims at correcting single point mutations. Chimeraplasts are chimeric molecules that are designed to contain the correct DNA sequence desired anda high affinity-binding region for that portion of the gene where the mutation is located. The chimeraplasts are
designed to preferentially bind to the mutated DNA region and to replace the incorrect nucleotide base with the correct one. This technique has been successfully used to “repair” the β-globulin point mutation for hemoglobin-S that causes sickle cell anemia. Between 10% and 20% of the genetic mutations in the blood samples tested were corrected and this is sufficient for relief of symptoms. It is possible that almost any single point mutation can be corrected by a specific chimeraplast. Other potential applications are the correction of p53 mutations in cancer cells, treatment of cystic fibrosis and of Duchenne muscular dystrophy.

C. Cell-Mediated Gene Therapy

This technique involves the genetic manipulation of cells followed by their in vitro amplification and subsequent injection of the modified cells into target tissues. For human gene therapy, success of cell-mediated methods depends on the capacity of cells to proliferate during the stage of in vitro amplification. One of the limitations is the well-known phenomenon of senescence of the diploid cells after a number of divisions. Several types of cells have been used for cell-mediated gene therapy:

**Fibroblasts:** These are located throughout the body and have an extended capacity for self-renewal. Fibroblasts have been used for the introduction of the following genes:

- Human Growth hormone
- Neurotrophic factors
- Adenosine Deaminase
- Glucocerebrosidase
- Factor IX
- Low Density Lipoprotein (LDL) receptors

Advantages of use of fibroblasts in gene therapy are:

- They are readily obtained by skin biopsy from the patients
- They are easy to culture
- Fibroblasts undergoing division in cultures are easily and stably transduced with retroviruses.

A major disadvantage of fibroblasts for gene therapy is that there is loss of gene expression in vivo due to apoptosis. Experiments in rats have shown the feasibility of gene transfer by genetically modified fibroblasts, which secrete NGF. Tusznyski et al (1996) have used retroviral vectors to genetically modify fibroblasts ex vivo to secrete NGF. These cells were then grafted to basal forebrain region of adult rhesus monkeys after fornix transection (model of Alzheimer’s disease) for periods up to 8 months. Recipients of NGF-secreting grafts showed a persistence of 70.4% of neurons whereas no NGF was detected in tissues from control grafts.

**Keratinocytes:** These are differentiated epidermal cells and have been used for insertion of various therapeutic genes in vitro using retroviral vectors. Keratinocytes are potentially appealing vehicles for delivery of secreted gene products because they can be transferred to human skin by the relatively simple procedure of grafting. Genetically engineered keratinocytes have been shown to incorporate into the healing of experimental wounds in animals suggesting the possibility of introducing other genes expressing therapeutic proteins into wounds to affect healing. Andree et al (1994) have described the acceleration of wound repair in pigs, on gene transfer into the wound of human epidermal growth factor (EGF) gene. This transfer is achieved with the use of particles coated with the plasmid and bombardment of the wound with “gene gun”.

A.1. Vectors for Gene Therapy

These can be divided into two broad categories:

1) The viral vectors (naturally occurring)
2) The non-viral vectors (artificial)

Naturally occurring vectors can be transmitted between different cells via three different mechanisms: conjugation (and mobilization), transduction, and transformation. Conjugation requires a plasmid, which must multiply in the domain cell so that it can be transferred to a recipient cell. For mobilization, a helper plasmid must make the necessary enzymes available. Transduction is based on viral DNA molecules, which are integrated into the genome of the cell and multiplied by cell division. Transfection is a passive process in which DNA is transported into the cell by physical or chemical means.
Obstacles to gene delivery:
- Delivery to the target cell may be hindered by barriers such as serum inactivation
- Attachment and entry into cells may be hindered by reduction or absence of receptors
- Release into the cytoplasm may be hindered by lysosomal degradation
- Entry into the cell nucleus may not occur due to lack of mitosis (required for retroviral vectors)
- The final step of expression may not occur adequately even if integration does occur

A.1.1. Viral Vectors

Although the concept of gene therapy and viral vectors are recent, vaccination such as for smallpox involved transfer of “foreign” genetic material into patients. Of all the methods of gene transfer, viral vectors are the most frequently used in the ongoing clinical trials. An overview of the commonly used viral vectors is shown in the following table:

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Retroviral Vectors</th>
<th>Adenoviral Vectors</th>
<th>Herpes Simplex Viral Vectors</th>
<th>Adeno-Associated Viral Vectors</th>
<th>Vaccinia (Pox) Viral Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert Capacity</td>
<td>8 kb</td>
<td>7-8 kb</td>
<td>30 kb</td>
<td>4.5 kb</td>
<td>&gt; 30 kb</td>
</tr>
<tr>
<td>Integration</td>
<td>Yes</td>
<td>Occasional</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Tissue specificity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Properties</td>
<td>Infects only dividing cells</td>
<td>Infects non-dividing cells</td>
<td>Neurotropic</td>
<td>Integrates in non-dividing cells</td>
<td>Wide host range used as vector for immunization</td>
</tr>
<tr>
<td>Administration</td>
<td>Ex vivo or direct injection</td>
<td>Ex vivo or direct injection, aerosolization</td>
<td>Ex vivo or direct injection</td>
<td>Probably ex vivo only</td>
<td>Direct skin scarification</td>
</tr>
<tr>
<td>Titer</td>
<td>(10^7)-(10^8) cfu/mL</td>
<td>(10^5)-(10^6) cfu/mL</td>
<td>(10^5)-(10^6) cfu/mL</td>
<td>(10^5)-(10^6) cfu/mL</td>
<td>(10^2)-(10^7) cfu/mL</td>
</tr>
<tr>
<td>Duration of Transgene Expression</td>
<td>Good</td>
<td>Transient</td>
<td>Transient</td>
<td>Potentially Good</td>
<td>Transient</td>
</tr>
<tr>
<td>Level of Transgene Expression</td>
<td>Moderate</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Safety Issues</td>
<td>Insertional Mutagenesis</td>
<td>Inflammatory response, Insertional Mutagenesis</td>
<td>Insertional Mutagenesis</td>
<td>Toxocity of rep protein, Insertional Mutagenesis</td>
<td>Dangerous in immunosuppressed patients</td>
</tr>
</tbody>
</table>

A.1.2. Nonviral Vectors for Gene Therapy

Due to some concern with the use of viruses as vectors, search has been made for alternative methods of gene therapy. In that case, DNA is delivered to the tissues “naked” or conjugated with carriers such as liposomes. The advantages of nonviral methods over the use of viral vectors are as follows:
- There is no limitation to the size of DNA that can be transferred
- DNA can be easily manipulated without special requirements for viral sequences required for packaging and replication.
- Less safety concerns as the constructs are unlikely to induce any infection
- There are less problems with immunogenicity

However, there are also major drawbacks with their use, such as the lack of tissue specificity and the much lower efficiency as compared to those of the viral vectors. Some characteristic examples of nonviral vectors are:
- Lipoplexes. These are complexes made of the highly negatively charged DNA with highly positively charged cationic liposomes.
- Negatively charged or pH-sensitive liposomes. These liposomes contain in their aqueous milieu the DNA.
- Ligand-Polylysine-DNA complexes. In its best example – Polyethylenimine[PEI] – it has been shown to provide exceptionally high levels of transgene expression in the mouse brain. Widespread expression of both marker and functional genes was obtained in neurons and glia following injection into the cerebral cortex, hippocampus and the hypothalamus. Transgene expression persisted for more than 3 months in cortical neurons. This vector has a potential for gene therapy of brain disorders.
- Receptor-Mediated Endocytosis. To circumvent the limitations of direct introduction of DNA by nonviral methods, techniques have been developed to deliver DNA via receptor-mediated pathway. The concept is illustrated in the following schematic figure:

![Schematic Diagram](image)

These vectors, called molecular conjugate vectors, use the internalization mechanism intrinsic to specific macromolecules. This involves targeting of genes to specific cell surface receptors such as transferring receptors. DNA is linked to a targeting molecule such as polylysine and the conjugate-DNA complex would bind to a specific cell surface receptor, inducing endocytosis and transfer of DNA into the cells.

- Artificial (Synthetic) Viral Vectors. The aim of constructing a viral vector is to eliminate unnecessary and harmful viral functions while retain the ability to infect a cell. The challenge is to develop and bring all of the components in a manufacturable form that has the requisite pharmacodynamic and pharmacokinetic profile in vivo. This may require additional factors such as polymers to ensure stability and to mask sites capable of eliciting immunological responses or contributing to degradation and clearance.

- Artificial Chromosomes. A theoretically desirable vector for gene therapy would be one wholly composed of native mammalian chromosomal components, including origins of replication, telomeres and a centromere. Advantages of Mammalian Artificial Chromosomes (MAC) include the following:
  - Large insert size
o Nonintegrative DNA construct
o MACs are autonomous replicating units and can bring about long-term expression during gene therapy
o Mitotic segregation without viral enzymes
o Potential delivery by molecular conjugate vectors

D. Routes of Administration and Target Tissues for Gene Therapy

These are summarized in the following tables as follows:

Potential routes of administration of DNA:

- Subcutaneous DNA Injection
- Intramuscular DNA Injection
- Direct DNA Injection into lesions such as tumors
- Intravenous DNA Injection
- Intraarterial delivery by catheter
- Intracerebral DNA Injection
- Intranasal DNA Injection
- Transmucosal delivery of DNA
- Intratracheal gene transfer
- Intra-organ DNA Injection: liver, kidney, lung
- Intraperitoneal DNA Injection
- Intramarrow gene transfer

Target tissues for gene therapy:

- Bone Marrow
- Epithelial cells: Gastrointestinal and respiratory systems
- Hepatocytes
- Kidney
- Peritoneum
- Muscle
- Neurons
- Ocular tissues
- Pancreas
- Skin
- Vascular Endothelium (mainly for prevention of restenosis in Cardiovascular Disorders)

A.1.3 Antisense Therapy

The basic mechanism of the antisense approach is to block the synthesis of cellular proteins by interfering with either the transcription of DNA to mRNA, or the translation of mRNA to proteins. There are two types of macromolecules that fall in the category of antisense therapy: the oligodeoxynucleotides and the ribozymes.

- An oligodeoxynucleotide (ODN) is a synthetic piece of DNA that is complementary to RNA or DNA of the genome or an RNA derived from it. All ODN’s should contain at least 15 nucleotides. These compounds prevent or disable the expression of disease-associated proteins.
- Ribozymes, enzymes comprised of RNA, catalyze RNA cleavage.

Antisense oligonucleotides may interfere with gene expression by the following mechanisms:

- Hybridization of the antisense oligonucleotides to the site of initiation of translation. This can result in the formation of a stable double-stranded structure, which cannot be resolved by the small subunit of the ribosome that scans for the starting codon AUG. As a result, the large subunit of the ribosome cannot assemble correctly with its small subunit and initiate translation.
- Binding of an antisense oligonucleotide to an RNA sequence. This results in the formation of an RNA:DNA duplex, the RNA portion of which can be cleaved by the enzyme Rnase H. Once cleaved, mRNA is no longer capable of translation and is rapidly degraded. The inhibition of this mechanism is irreversible, but it lacks specificity.

- Protein binding. ODNs can bind to several proteins with satisfactory affinities. It has been shown that an ODN containing 28 consecutive cytidine residues can block the binding of gp120 to CD4. HIV-1 replication is inhibited by this mechanism.

- Sequence-specific antisense independent effects of ODNs as follows:
  - ODNs can bind outside the cell to growth factors such as bFGF thereby inhibiting cell proliferation
  - ODNs may bind to cellular receptors such as CD4 and interfere with entry of HIV-1 into the cell.
  - ODNs may be degraded by lysozyme to cytotoxic nucleosides, which can interfere with signal transduction processes by binding to protein kinase C.

ODNs bear Phosphorothioate bonds instead of the naturally occurring Phosphodiester ones. This sulfur substitution for the nonabridged oxygen at the internucleotide linkage makes them nuclease-resistant, as opposed to the Phosphodiester ones, that are rapidly degraded as they enter the cytosolic environment.

The mode of action of Ribozymes as a means of antisense therapy is demonstrated in the following scheme:

As it is apparent from the figure, this ribozyme approach applies to a possible viral infection. Specifically, the therapeutic potential of ribozymes is tailored for undesirable expression of RNA, such as viral infections and neoplastic disorders, plus some genetic diseases resulting from aberrant splicing of pre-mRNAs.

The use of ribozymes for HIV infection has been well recognized. HIV has an RNA genome with several potential ribozyme cleavage sites along the length of the viral genome and subgenomic RNAs. Because the virus mutates rapidly, it becomes resistant to antiviral drugs, which inhibit a single viral target. Multiple ribozymes which can be targeted to a number of different sites simultaneously to inhibit HIV-1 have become an important alternative treatment. Ribozymes can be effective against HIV-1 infection at two points in the life cycle of the virus: The first is immediately following infection
prior to proviral DNA formation, when all or part of the viral genome is still in the form of RNA. The second is following the establishment of integrated provirus from which spliced and full length transcripts are produced.

Certain types of neoplasias are definite targets of ribozymes as a therapeutic approach, based on the fact that production of aberrant proteins with the capacity to transform cells is a key feature in these disorders. Potential targets for ribozyme-mediated cleavage as an approach against neoplastic diseases include the following:
- Fusion transcripts: bcr/abl
- Transcripts of regulating genes: cdk2
- Transcripts of antiapoptotic genes: bcl2,bcl-x
- Transcripts of cytostatic resistance genes: MDR-1, topoisomerases
- Nuclear transcription factors: c-myc, c-fos, c-jun

E. Immunogene Therapy

A merger of molecular biology techniques, advanced drug delivery and immunology has provided medicine with tools to ward off infectious disease and possibly treat autoimmune disorders and cancer. The term immunogene therapy means the use of genes for immunotherapy, and gene-based vaccines form a major portion of this approach.

E.1. DNA-Based Vaccines

These are composed of nonreplicating DNA plasmids encoding some genes from pathogenic organisms or tumor cells. Such vaccines are capable of inducing protective antibody and cell-mediated immune response against a variety of organisms. In case of viral vaccines, host cells take up the foreign DNA, express the viral gene, and make the corresponding viral protein inside the cell. In a naked DNA vaccine, the DNA has been freed of all the proteins in the usual DNA-protein complex. Naked DNA vaccines derived from plasmids could bypass the numerous problems associated with other vectors such as immune response against the delivery vector. Advantages of DNA vaccines are:
- Elimination of the threat of introduction of a potentially virulent virus associated with “attenuated” vaccines
- Large scale production of DNA can reduce the price of a final product greatly
- DNA can be stored in a dry powdered for years and still retains its activity
- Low doses of a proper gene construct can induce protective immunization
- A single application can lead to long-lasting immunity, eliminate the need for booster doses and increase compliance

Applications of DNA vaccination are listed in the following table:

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<td>- DNA vaccine for tuberculosis</td>
<td>Genetic immunization against allergen-induced disorders</td>
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<tr>
<td>- Influenza</td>
<td>- DNA vaccine for malaria</td>
<td>DNA immunization for autoimmune diseases</td>
</tr>
<tr>
<td>- Herpes simplex</td>
<td>- DNA vaccine for Leishmania sis</td>
<td></td>
</tr>
<tr>
<td>- AIDS</td>
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<td></td>
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<td>- Rabies</td>
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</tr>
<tr>
<td>- Rabies</td>
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<td></td>
</tr>
</tbody>
</table>
E.2. DNA Vaccine for Tuberculosis

Immunization with DNA encoding a mycobacterial antigen provides an efficient and simple method for generating protective immunity. Tascon et al (1996) have demonstrated that when mice were injected with a single mycobacterial antigen (heat shock protein 65) they became immune to subsequent challenge with *Mycobacterium tuberculosis*. Protection was equal to that achieved by vaccinating with BCG.

E.3. Cancer Vaccines

DNA vaccination can be used for tumors induced by viruses:

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human T-cell leukemia virus (HTCLV-1)</td>
<td>Adult T-cell leukemia (ATL)</td>
</tr>
<tr>
<td>Herpes-like viruses</td>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td>Human Papilloma virus (HPV)</td>
<td>Lymphoms in AIDS</td>
</tr>
<tr>
<td>Epstein-Barr virus (EBV)</td>
<td>Cervical cancer</td>
</tr>
<tr>
<td>Hepatitis B virus (HBV)</td>
<td>1. Immunoblastic lymphoma</td>
</tr>
<tr>
<td></td>
<td>2. Nasopharyngeal carcinoma</td>
</tr>
</tbody>
</table>

About 20% of human cancers have a viral etiology. Incidence of such cancers, however, is lower than the frequency of the viral infections indicating mechanisms other than the presence of virus. Preventive vaccination directed against the virus may reduce the tumor incidence because viral antigens can be clearly defined and can be recognized as foreign, eliciting the production of neutralizing antibodies. The best way of killing cancer cells is by the action of cytotoxic cells. The cytolytic CD8+ T-cell is an ideal candidate for this purpose. Some cancer vaccines are shown below:

<table>
<thead>
<tr>
<th>Antigenic Target</th>
<th>Tumor</th>
<th>Method of Administration</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinobryonic antigen (CEA)</td>
<td>Cancer Colon</td>
<td>Intramuscular</td>
<td>In clinical trials</td>
</tr>
<tr>
<td>Idiotype on the surface of the B-lymphoma cell</td>
<td>B-cell Lymphomas</td>
<td>Intramuscular</td>
<td>Encouraging results in clinical trials</td>
</tr>
<tr>
<td>MAGE 1 (Melanoma Associated Gene) Antigen</td>
<td>Melanoma</td>
<td>Intramuscular, Intra-/Subcutaneous, intravenous</td>
<td>Aiming to booster highly specific MHC class-I pathways for induction of tumor reactive CD8+ CTLs</td>
</tr>
<tr>
<td>p210 BCR-ABL</td>
<td>Chronic Myelogenous Leukemia (CML)</td>
<td>Intramuscular</td>
<td>Antigen is a mutated oncogene product</td>
</tr>
<tr>
<td>Mutant p53</td>
<td>&gt;50% of human tumors</td>
<td>Intramuscular</td>
<td>Antigen is a mutated tumor suppressor</td>
</tr>
<tr>
<td>HPV 16 E7</td>
<td>Cervical Cancer</td>
<td>Intramuscular</td>
<td>Antigen is a viral gene product</td>
</tr>
<tr>
<td>PSA (Prostate Specific Antigen)</td>
<td>Cancer of the Prostate</td>
<td>Intramuscular</td>
<td>Antigen is a tissue-specific self-antigen</td>
</tr>
</tbody>
</table>
F. Gene Therapy for Genetic Diseases

Monogenic diseases (about 4,000 of them) were the first targets considered for gene therapy because the role of alterations in DNA was evident, the consequences were severe and there were few, if any, alternative therapies in many cases. Although the underlying genetic defect is clearly present in all the cells of the body, there are diseases in which correcting the defect in only one given tissue might suffice to improve or even normalize the patient's clinical status. Some examples are Gaucher's disease (glucocerebrosidase deficiency) mucopolysaccharidosis (beta-glucuronidase deficiency), hyperammonemia (ornithine transcarbamoylase deficiency) and adenosine deaminase deficiency. Adenosine deaminase (ADA) deficiency was one of the first diseases to be targeted for gene therapy. Several trials are currently ongoing with some degree of success. ADA deficiency is a rare (1/100 000) and fatal disease characterized by the inability of affected individuals to mount a normal immune response. This results in recurrent severe opportunistic infections leading to death in the first years of life. Twenty-five per cent of cases of severe combined immunodeficiency (SCID) are due to an abnormality of the gene coding for the enzyme ADA. When this enzyme is inactive, cells accumulate deoxyadenosine and its derivatives (in particular ATP), which attain toxic levels and appear to block the synthesis of DNA or even destroy cells. T cells have a higher turnover of deoxyadenosine than other cells and are therefore more vulnerable. The result is severe cell-mediated immunodeficiency with T-lymphopenia, low levels of total immunoglobulin and defective specific antibody responses, accounting for the extreme sensitivity of ADA-deficient patients to opportunistic infections. Bone marrow allografting is the ideal curative treatment, resulting in almost 100% cure rate. But only 30% of patients are fortunate enough to find an HLA-identical donor. The gene therapy protocol for this disease is simply an \textit{ex vivo} transfer of ADA gene to T lymphocytes of the patient. After the reinfusion of the transfected T cells back to the patient, their number as well as the cellular and humoral responses normalized. Gene treatment ends after about two years with integration of the vector and persistence of the ADA gene.

G. Cancer Gene Therapy

Cancer is the most common cause of death in the developed countries. About 2.5 million cases of cancer are diagnosed worldwide every year. In males, the most common neoplasia is the one of the prostate, while in females of the breast. Lung cancer ranks second in both sexes. Cancer protocols (both marker and therapy) constitute about 70% of all current gene therapy protocols. These protocols may be classified into several categories:

1) DNA based cancer vaccines (vide supra)
2) Transfer of suicide genes, which confer drug sensitivity to cancer cells and induction of bystander killing effect. Transduced cells become highly sensitive to the corresponding drug and can be selectively eliminated
3) Transfer of antisense genes to block the expression of deleterious cancer-promoting genes
4) Transfer of drug resistance genes into non-malignant blood stem cells to protect them from subsequent chemotherapy. Once physiological stem cells are protected, a more aggressive chemotherapy can be attempted to eliminate tumor cells
5) Transfer of tumor suppressor genes into cancer cells to replace a missing or damaged cancer-blocking gene
6) Transfer of recombinant antibody genes to interfere with tumor cell specific functions
7) Transfer of genes coding for proteins, which boost the host's antitumor immune response. Nabel's protocol was the first to show that transferring the expression of allogeneic MHC proteins to in situ tumors increased tumor rejection. Additionally, CTLs specific to the untransduced autologous tumor may be generated by this immunotherapy
8) Gene therapy targeting the tumor vasculature

Suicide gene therapy is based on introducing a drug sensitivity gene into target cells, which are then killed by the drug at doses that are not detrimental to normal cells. Most suicide genes currently under investigation mediate sensitivity by encoding viral or bacterial enzymes that convert inactive forms of a drug into toxic metabolites capable of inhibiting nucleic acid synthesis.
Herpes Simplex Virus- thymidine kinase (HSV-tk): This is the best example of a suicide gene. This gene encodes for the enzyme thymidine kinase, which catalyzes the phosphorylation of the antiviral drug ganciclovir (and aciclovir). When ganciclovir is administered, it is taken up by the cells and those expressing the HSV-tk they convert the monophosphate to the triphosphate form of the drug. The latter is 1000 times more toxic than the generic ganciclovir, inhibiting the association of deoxynucleoside triphosphates with DNA polymerase, and inducing DNA chain termination. HSV-tk gene transfer forms the basis of gene therapy for malignant gliomas. The tumor cells are vulnerable to antiviral drug ganciclovir given systemically, while the HSV-tk gene has been administered by a local intratumor injection. The tumors are eliminated in animal models despite a less than 100% gene transfer efficiency. This phenomenon is referred to as the “bystander effect” and allows the destruction of neighboring cells. The presence of this bystander effect is important in achieving complete regression of brain tumors in experimental animals because none of the viral vectors used for therapy of brain tumors are able to transduce 100% of the tumor cells in the brain. The mechanism of the bystander effect has not been elucidated yet; some of the possible explanations are listed below:

- According to the leading hypothesis there is transfer of phosphorylated ganciclovir moieties from HSV-tk positive cells to HSV-tk negative cells through gap junctions. Normal brain tissue is protected because of obliteration of gap junctions at the interface of tumor and normal tissues. Concern has also been expressed about possible development of resistance to ganciclovir by this approach
- Cell fragments from cells destroyed by HSV-tk/ganciclovir are taken up by neighboring cells by phagocytosis and cause death of these cells
- Direct cell-to-cell transfer of phosphorylated ganciclovir moieties through apoptotic vesicles
- An immune response to ganciclovir metabolites

2) Vide supra
3) MDR (Multi-Drug Resistance) gene can be introduced into Human Hematopoetic Stem Cells (HSCs) to reduce chemotoxicity and allow administration of higher doses of chemotherapy in cases of treatment of bone marrow neoplasias.
4) p53 is the best known of the tumor suppressor genes. The p53 encodes a nuclear protein, which binds to and modulates the expression of genes important for DNA repair, cell division, and cell death by apoptosis. Apoptosis has been linked to p53 function in some cell types; by the same token, loss of p53 function renders cells resistant to apoptosis in many cases. Mutations of p53 gene are the single most common genetic alteration observed in human cancers; a mutant p53 has been detected in nearly half of human cancers. The p53 is located on chromosome 17p13.1, which is one of the more frequent targets for chromosome alterations in human cancer. It is not required for normal development but lack of p53 function raises the risk of cancer enormously. (A gene with significant amino acid similarity to p53 has been identified recently and is called p73 (Jost et al, 1997)). It is located in the short arm of chromosome 1 and when overproduced, activates the transcription of p53-responsive genes and inhibits cell growth in p53-like manner by inducing apoptosis. Direct injection of a p53 adenovirus construct into experimentally induced tumors followed by intraperitoneal administration of cisplatin has been shown to induce massive apoptotic destruction of the tumors as compared to cisplatin treatment alone.
8) The role of angiogenesis in tumor growth is well recognized. Destruction of tumor vasculature may inhibit the primary growth and metastases. Blood vessels are not genetically altered in tumors and the problem of drug-resistance does not arise in the tissues of the vasculature. Tumor endothelium proliferates at a rate up to 100-fold that of the normal endothelium providing the possibility of differential toxicity. Ozaki et al (1996) have shown that endothelial cell-specific growth suppression is possible by transduction of the suicidal genes driven by a promoter sequence of the von Willebrand factor gene, which is widely expressed in many endothelial cells. This strategy is expected to kill the proliferating endothelial cells preferentially and may lead to inhibition of tumor angiogenesis, growth and metastasis \textit{in vivo}.

**G1. Gene marking and gene therapy**

The term "gene therapy" is now commonly used to designate somatic gene transfer. It does not necessarily assume a therapeutic effect, and may therefore be considered by some to be an improper usage of the term. It is, however, so widely used nowadays, that it has been adopted. In cases where the term is ambiguous the term "gene therapy" is used to indicate a procedure which aims to treat or even cure a patient, as opposed to "gene marking", where the gene transduced simply aims to track the fate of cells in the body with no expectation of a beneficial therapeutic effect.
NeoR/TIL Gene Marking

The basis of this protocol was the transfer of gene-marked immune cells (specifically tumor infiltrating lymphocytes, or TIL) into patients with advanced cancer. The protocol had two primary objectives:
- To demonstrate that an exogenous gene could be safely transferred into a patient and
- To demonstrate that the gene could be detected in cells taken back out of the patient.

The protocol asked a number of scientific and clinical questions, generated by several earlier experiments. In 1986, a clinical protocol for the treatment of advanced malignant melanoma with the newly discovered class of immune cells called TIL was initiated at the National Institutes of Health (NIH). These lymphocytes are T cells that are isolated directly from the tumor and they are grown to large numbers in tissue culture in the presence of interleukin-2 (IL-2). After expansion in culture several thousand times, approximately \(2 \times 10^{11}\) TIL are given back to the patient intravenously in addition to high doses of IL-2 in several days of treatment. Even in those patients who did not respond to all other therapy (including treatment with IL-2 alone), 40% of patients responded to this protocol. The large-scale tissue culture and the large number of cells and IL-2 that are given to the patient make this procedure expensive and clinically difficult. Furthermore, 60% of patients failed to respond to this treatment, and even those who did, the response was transient (6-12 months). It is likely that only a subset of the heterologous population of cells administered to a patient are effective in killing cancer cells \(in vivo\); one goal of investigators is to determine which these cells are. It would be useful to be able to follow and study the administered cells in the patient’s body to learn where they go and how long they survive. One approach would be to mark the TIL so that they could be tracked in the body. In 111 has a half-life of just 2.8 days, so that only short-term data could be obtained. The NeoR/TIL clinical protocol proposed to take an aliquot of cells from TIL early in their culture, transfer into them a marker gene (the neomycin resistance gene NeoR obtained from Escherichia coli) with a retroviral vector, grow the marked cells in parallel with the unmarked cells, and then give both populations back to the patient. Periodic blood samplings would indicate how long the TIL survived in the bloodstream, periodic tumor biopsies would indicate if and roughly how many marked TIL were present in the tumors, and if enough patients could be studied, perhaps a correlation could be drawn between the presence of marked TIL and the clinical response.

H. Prospects for \textit{in Utero} Gene Therapy

Gene therapy for the treatment of disease in children and adults is being actively pursued at many medical centers. However, a number of genetic disorders result in irreversible damage to the fetus before birth. In these cases, as well as for those with genetic diseases who may benefit from therapy before symptoms are manifested, \textit{in utero} gene therapy (IUGT) could be beneficial. Although some successes with in utero gene transfer have been reported in animals, significant questions remain to be answered before IUGT clinical trials would be acceptable.

\textbf{Rationale for Human in Utero Gene Therapy}

The rationale for human IUGT is that it may allow the correction of some types of genetic diseases before the appearance of any clinical manifestations; in addition, gene transfer in the fetus offers a number of potential advantages over postnatal gene transfer (see below). For the neurologic genetic diseases (such as Tay-Sachs, Niemann-Pick, Lesch-Nyhan, Sandhoff, Leigh, many leukodystrophies, generalized gangliosidosis) that appear to produce irreversible damage during gestation, treatment before birth (perhaps early in pregnancy) may be required to allow the birth of a normal baby. Unfortunately, although these neurologic diseases may appear to be the logical targets for attempts at IUGT, it is not known how efficiently and safely target brain tissue either in the adult or in the developing fetus. In some cases, gene transfer into blood cells, some of which will become microglial cells in the brain, may provide an approach for treatment. Initially, it will probably be necessary to target diseases that could be treatable by inserting a therapeutic gene into a more accessible cell type, specifically the hematopoietic stem or progenitor cell (HSC). Diseases that might be targeted in this way include immunologic diseases (for example, severe combined immunodeficiency), hematologic diseases (for example, thalassemias), and metabolic diseases (for example, osteopetrosis). Although a broad range of cell types is being engineered in the various gene therapy protocols, the target that appears most promising for IUGT is the HSC because of the high proliferative potential and multilineage differentiation potential of HSCs for delivering the corrective gene to the patient. A number of viral and nonviral vectors have been used in gene therapy protocols. However, only retroviral vectors integrate efficiently into the target cell’s genome and therefore insert the therapeutic gene permanently into the genetic make-up of the cell. For genetic diseases, where correction for the lifetime of the patient is desired, only retroviral vectors...
appear suitable at this time. A number of existing clinical protocols are based on ex vivo retroviral vector transduction of HSCs, followed by transplantation of the gene-engineered cells back into the patient. The primary disadvantage of IUGT is the potential risk of harm to both the fetus and the mother. Advances in prenatal diagnosis and molecular analysis now allow the identification of many congenital disorders by evaluation of trophoblastic tissue obtained by chorionic villus sampling (CVS) at 8 to 10 weeks of gestation. High-resolution ultrasound and midgestational interventional techniques have developed to the point that the manipulative techniques necessary to carry out second-trimester gene transfer at low risk to the mother and fetus are now available. However, there are a number of other risks inherent to in utero gene transfer, including possible interference with the developmental process from insertional mutagenesis and potential germ line gene transfer.

**Advantages of IUGT**

For a number of genetic diseases it may be advantageous to use IUGT rather than to wait until after birth to begin treatment. First, successful early treatment could preempt the appearance of any clinical manifestations of a disease. Second, gene transfer in the fetus is believed to be more efficient than in the more mature organism (vide infra), so that gene therapy should be easier to accomplish prenatally than postnatally. Third, immunological naïveté and the permissive environment of the early gestational fetus should allow acceptance of cells and vector without the need for immunosuppression or myeloablation. In early immunologic development, before thymic processing of mature lymphocytes, the fetus appears to be largely tolerant of foreign antigens. Exposure to foreign antigens during this period often results in sustained tolerance, which can become permanent if the presence of the antigen is maintained; transplantation of allogeneic or xenogeneic HSCs results in the creation of permanent chimeras. Cellular tolerance appears to be secondary to clonal deletion of reactive lymphocytes in the thymus, whereas the mechanism of B-lymphocyte tolerance (peripheral tolerance) appears to involve both clonal deletion and clonal suppression. The end result is an immune system that is specifically tolerant of foreign antigenic sources. The possible development of tolerance to the vector and gene product may permit postnatal treatment of the patient, if required, with relative safety.

The developing fetal hematopoietic system provides additional advantages that can help circumvent some of the major difficulties encountered with postnatal treatment. The naturally occurring transition in the primary site of hematopoiesis from yolk sac to liver and spleen and finally to bone marrow during ontogeny is accomplished by the migration of HSCs from one site (liver) to another (bone marrow) via the circulation. Fetal circulation can serve as a source of large numbers of HSCs for IUGT that are destined to populate the developing bone marrow system of the fetus. Furthermore, the availability of bone marrow spaces for homing and engraftment of HSCs in the fetus allows for the engraftment of transduced HSCs without the need for cytoablation of the patient’s own marrow, thus avoiding the risks associated with this procedure. Finally, fetal HSCs (as well as many other cell types) are rapidly dividing in order to provide cells to the growing organism so that they are much better targets for retrovirally mediated gene transfer than adult HSCs.

**Safety Issues**

*The mother.* The additional risk created by adding a gene transfer component to standard obstetrical procedures should be minimal for the mother. However, there can be unique risks such as accidental transfer of vector into the maternal blood stream. On the basis of the studies in sheep, this risk is probably very low; the amount of transfer into ewes was minimal and transient. Nonetheless, there is evidence that placental tissue is readily infected with a variety of retroviruses and that the placenta allows the transfer of retroviral-like particles from the mother to the fetus. Carefully designed animal studies are needed to fully address this question, especially in cases where multiple injections of high-titer vector may be contemplated.

Of greater significance is the possible harm to the mother if a fetus with a lethal disease, like homozygous [alpha]-thalassemia, is only partially treated. The thalassemic fetus usually dies during the third trimester, producing toxicity for the mother. These fetuses are ordinarily aborted at 24 weeks for the protection of the mother. A serious situation would exist if a fetus were to be kept alive by IUGT but only in critical condition, thereby inducing ongoing toxicity in the mother and, perhaps, the birth of a severely ill, destined-to-die infant. The in utero approach must result in sufficient gene expression to ensure that the affected fetus is significantly benefited. Experiments in large animal models are needed to provide quantitative assessments of the amount and duration of transgene expression.

*The fetus.* For direct intraperitoneal injection of vector in the 13- to 15-week fetus, the risks of the procedure are low because of the technological advances that have been made in obstetrical techniques. For gene-engineered HSC transplantation, the risks would be similar to those for HSC transplantation, except that autologous HSCs would be used, thereby adding the risk of the initial collection. By week 18, fetal blood collection in experienced hands is low risk. Experience with in utero treatment for Rh disease would indicate a 1 to 3.5% risk factor. Nonetheless, even though the extensive obstetric experiences with CVS and fetal transfusion can be...
informative, the special circumstances of removal of blood from the fetus and its reinjection into the fetus will require additional studies in human-sized animals to determine risks. In addition, there are the as-yet-unknown risks associated with insertional mutagenesis that could lead to possible interference with developmental processes or to tumor formation. This concern is particularly acute with the direct injection approach, because there would be widespread distribution of the vector throughout the fetus. When it becomes possible to target vector to specific cell types, then this concern will lessen. These problems have not been apparent when in utero gene transfer occurred after the first trimester in the small and large animals studied, but more data are needed to determine the actual amount of risk.

Inadvertent germ line alteration. There is no biological basis to indicate that the cellular approach to IUGT would result in germ line alterations, because only transduced cells, and no retroviral vector, are given to the fetus. However, the possibility that the direct vector injection protocol may inadvertently lead to the alteration of the germ line cannot be ignored. Studies in mice and sheep have thus far failed to indicate any detectable transfer of exogenous genes into germ line cells when in utero gene transfer occurs after the first trimester. In a carefully designed study, 578 offsprings were evaluated of matings in which either one or both parent mice were injected with a high dose of vector (in this case, an adenoviral vector). The dose of the vector was sufficient to affect 80% of the hepatocytes with a small amount of dissemination to ovaries and testes in 94% of the animals. No evidence of germ line transmission was seen. In sheep, breeding studies (in which either one or both parents expressed in utero-administered transgene activity) produced 10 lambs. None of the offspring exhibited proviral DNA. In addition, although ejaculates were on occasion positive by polymer chain reaction (PCR) analysis, further PCR analysis of the purified sperm obtained from seven treated rams on a total of 21 separate occasions demonstrated no proviral DNA.

Conclusions
The IUGT approach is promising not only for the treatment of genetic diseases that produce fetal damage before birth, but also as an additional treatment procedure that may offer advantages over postnatal therapy. However, despite the existence of encouraging data in small and large animals, the question remains whether an IUGT protocol can be developed that will provide sufficient gene transfer in vivo to be effective in the treatment of a genetic disease. Once questions concerning the appropriate disease, the delivery procedure, efficacy, safety, and germ line transmission are successfully resolved by further animal experiments, then it would be appropriate to conduct clinical trials using IUGT.

Questions

1.) In some protocols, the p53 mutation has been corrected by inserting the gene utilizing a retrovirally-mediated transport. However, this did not have any effect on the course of the malignancy. How do you explain that?

2.) Suppose that a disease is being corrected by gene therapy, in which a surface protein was originally missing (e.g. a channelopathy). Would that lead to a formation of antibodies against this newly expressed protein, as it is “foreign” to the organism? How could that problem be bypassed?

3.) About 10 years ago, a company cloned the CD4 of the T cells upon which the HIV virus attaches, in order to “trick” the virus on which CD4 chains it should bind to. All patients who received the purified CD4 glycoprotein chains died within a few days and the company was sued for millions of dollars by the relatives of the diseased persons. How do you explain these deaths?

4.) The effectiveness of a chemotherapy in case of a chemosensitive malignant tumor is approximately 99.97%. Why isn’t that enough to eliminate the tumor? Why gene therapy is theoretically a more effective approach against malignancy?
References

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Links on the World Wide Web concerning Gene Therapy:

The Institute for Human Gene Therapy: http://www.med.upenn.edu/hgt/
GeneBrowser: http://www.natx.com/
NIH: http://www.nih.gov/od/oba/
GeneMed Network: http://genemed.org/
The Journal of Gene Medicine: http://www.wiley.co.uk/genetherapy/clinical/related-sites.html