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GENE THERAPY: THE FOREFRONT OF MEDICINE

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The concept of transferring genes to tissues for clinical applications has been discussed for nearly half a century, but the ability to manipulate genetic material via recombinant DNA technology has brought this goal to reality. While originally conceived as a way to treat life-threatening disorders (inborn errors, cancers) refractory to conventional treatment, gene therapy is now considered for many non–life-threatening conditions, including those adversely affecting a patient's quality of life. The lack of suitable treatment has become a rational basis for extending the scope of gene therapy. This manuscript reviews the general methods by which genes are transferred as well as diverse examples of clinical applications (acquired tissue damage, upper gastrointestinal tract infection, autoimmune disease, systemic protein deficiency). Despite some well-publicized problems, gene therapy has made substantive progress, including tangible success, albeit much slower than was initially predicted. Although gene therapy is still at a fairly primitive stage, it is firmly science based. There is justifiable optimism that with increased patho-biological understanding and biotechnological improvements, gene therapy will become a standard part of clinical practice within 20 years.

Gene therapy involves the insertion of a functioning gene into cells to correct a cellular dysfunction or to provide a new cellular function. This technology can be used to correct or replace the defective genes responsible for causing disease. Gene therapy has been especially successful in the treatment of diseases such as cystic fibrosis, combined immunodeficiency syndromes, muscular dystrophy, hemophilia etc. showing lasting and remarkable therapeutic benefit (Sheridan,2011).

However, it is important to remember that gene therapy is not a new idea. In 1963, Joshua Lederberg wrote, "We might anticipate the . . . interchange of chromosomes and segments. The ultimate application of molecular biology would be the direct control of nucleotide sequences in human chromosomes, coupled with recognition, selection and integration of the desired genes. . . . It will only be a matter of time . . . before polynucleotide sequences can be grafted by chemical procedures onto a virus DNA." Less than 30 years later, the first clinical study using gene transfer was reported. Rosenberg and his colleagues used a retroviral vector to transfer the neomycin resistance marker gene into tumor-infiltrating lymphocytes obtained from 5 patients with metastatic melanoma (Kohn and Candotti,2009). These lymphocytes then were expanded *in vitro* and later re-infused into the respective patients. Since this first study showed that retroviral gene transfer was safe and practical, it led to many other studies. Indeed, since 1989, more than 900 clinical trials have been approved worldwide. What made gene therapy possible between 1963 and 1990 was the development of recombinant DNA technology (Ferrua *et al*.,2010).

Types of Gene Therapy

Virtually all cells in the human body contain genes, making them potential targets for gene therapy. However, these cells can be

divided into two major categories: somatic cells (most cells of the body) or cells of the germline (eggs or sperm). In theory it is possible to transform either somatic cells or germ cells (Strachnan and Read, 2004).

Gene therapy using germ line cells results in permanent changes that are passed down to subsequent generations. If done early in embryologic development, the gene transfer could also occur in all cells of the developing embryo. The appeal of germ line gene therapy is its potential for offering a permanent therapeutic effect for all who inherit the target gene. Successful germ line therapies introduce the possibility of eliminating some diseases from a particular family, and ultimately from the population, forever. However, this also raises controversy. Some people view this type of therapy as unnatural, and liken it to "playing God." Others have concerns about the technical aspects (Anderson, 1985). They worry that the genetic change propagated by germ line gene therapy may actually be deleterious and harmful, with the potential for unforeseen negative effects on future generations.

Somatic cell therapy is viewed as a more conservative, safer approach because it affects only the targeted cells in the patient, and is not passed on to future generations (Culliton,1990). In other words, the therapeutic effect ends with the individual who receives the therapy. However, the effects of somatic cell therapy are short-lived. Because the cells of most tissues ultimately die and are replaced by new cells, repeated treatments over the course of the individual's life span are required to maintain the therapeutic effect. Transporting the gene to the target cells or tissue is also problematic. Regardless of these difficulties, however, somatic cell gene therapy is appropriate and acceptable for many disorders, including cystic fibrosis, muscular dystrophy, cancer, and certain infectious diseases. All gene therapy to date on humans has been directed at somatic cells, whereas germline engineering in humans remains controversial and prohibited in for instance the European Union (Krimsky, 1990).

Somatic gene therapy is again divided into two types:

Ex vivo, where cells are modified outside the body and then transplanted back in again. In such gene therapy procedure, cells from the patient's blood or bone marrow are removed and grown in the laboratory. The cells are exposed to the virus that is carrying the desired gene. The virus enters the cells and inserts the desired gene into the cells' DNA. The cells grow in the laboratory and are then returned to the patient by injection into a vein. This type of gene therapy is called ex vivo because the cells are treated outside the body (Hauser *et al*,2000).

In vivo, where genes are incorporated/altered in cells still in the body. This form of gene therapy is called in vivo, because the gene is transferred to cells inside the patient's body.

History of Gene Therapy

1970s and earlier

In 1972 Friedmann and Roblin authored a paper in Science with the title "Gene therapy for human genetic disease?". They cite 'Rogers S' for proposing that exogenous "good" DNA be used to replace the defective DNA in those who suffer from genetic defects. They also cite the first attempt to perform gene therapy as York Times, 20 Sept. 1970 (Friedmann and Roblin,1972).

2002 and earlier

New gene therapy approach repairs errors in messenger RNA derived from defective genes. This technique has the potential to treat the blood disorder thalassaemia, cystic fibrosis, and some cancers. Published as "Subtle gene therapy tackles blood disorder" at NewScientist.com (October 11, 2002) (Brown et *al*.,2006)

Researchers at Case Western Reserve University and Copernicus Therapeutics were able to create tiny liposomes 25 nanometers across that can carry therapeutic DNA through pores in the nuclear membrane. Published as "DNA nanoballs boost gene therapy" at NewScientist.com (May 12, 2002).

Sickle cell disease is successfully treated in mice. Published as "Murine Gene Therapy Corrects Symptoms of Sickle Cell Disease" from March 18, 2002, issue of The Scientist.

In 1992 Doctor Claudio Bordignon working at the Vita-Salute San Raffaele University, Milan, Italy performed the first procedure of gene therapy using hematopoietic stem cells as vectors to deliver genes intended to correct hereditary diseases. This was a world first. In 2002 this work led to the publication of the first successful gene therapy treatment for adenosine deaminase-deficiency (SCID) (Fisher *et al*.,2010).

The success of a multi-center trial for treating children with SCID (severe combined immune deficiency or "bubble boy" disease) held from 2000 and 2002 was questioned when two of the ten children treated at the trial's Paris center developed a leukemia-like condition. Clinical trials were halted temporarily in 2002, but resumed after regulatory review of the protocol in the United States, the United Kingdom, France, Italy, and Germany.

In 1993 Andrew Gobea was born with severe combined immunodeficiency (SCID). Genetic screening before birth showed that he had SCID. Blood was removed from Andrew's placenta and umbilical cord immediately after birth, containing stem cells. The allele that codes for ADA was obtained and was inserted into a retrovirus. Retroviruses and stem cells were mixed, after which they entered and inserted the gene into the stem cells' chromosomes. Stem cells containing the working ADA gene were injected into Andrew's blood system via a vein. Injections of the ADA enzyme were also given weekly. For four years T-cells (white blood cells), produced by stem cells, made ADA enzymes using the ADA gene. After four years more treatment was needed (Perez *et al*., 2008).

2003

In 2003, a research team of University of California inserted genes into the brain using liposomes coated in a polymer called polyethylene glycol (PEG). The transfer of genes into the brain is a significant achievement because viral vectors are too big to get across the blood-brain barrier. This method has potential for treating Parkinson's disease. Published as "Undercover genes slip into the brain" at NewScientist.com (March 20, 2003) (Urnov *et al*.,2010, Rezai *et al*.,2011).

RNA interference or gene silencing may be a new way to treat Huntington's disease. Short pieces of double-stranded RNA (short, interfering RNAs or siRNAs) are used by cells to degrade RNA of a particular sequence. If a siRNA is designed to match the RNA copied from a faulty gene, then the abnormal protein product of that gene will not be produced. Published as "Gene therapy may switch off Huntington's" at NewScientist.com (March 13, 2003).

2006

Scientists at the National Institutes of Health (Bethesda) have successfully treated metastatic melanoma in two patients using killer T cells genetically retargeted to attack the cancer cells. This study constitutes the first demonstration that gene therapy can be effective in treating cancer.

In March 2006 an international group of scientists announced the successful use of gene therapy to treat two adult patients for a disease affecting myeloid cells. The study, published in Nature Medicine, is believed to be the first to show that gene therapy can cure diseases of the myeloid system.

In May 2006 a team of scientists led by Dr. Luigi Naldini and Dr. Brian Brown from the San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) in Milan, Italy reported a breakthrough for gene therapy in which they developed a way to prevent the immune system from rejecting a newly delivered gene. Similar to organ transplantation, gene therapy has been plagued by the problem of immune rejection. So far, delivery of the 'normal' gene has been difficult because the immune system recognizes the new gene as foreign and rejects the cells carrying it. To overcome this problem, the HSR-TIGET group utilized a newly uncovered network of genes regulated by molecules known as microRNAs. Dr. Naldini's group reasoned that they could use this natural function of microRNA to selectively turn off the identity of their therapeutic gene in cells of the immune system and prevent the gene from being found and destroyed. The researchers injected mice with the gene containing an immune-cell microRNA target sequence, and spectacularly, the mice did not reject the gene, as previously occurred when vectors

without the microRNA target sequence were used. This work will have important implications for the treatment of hemophilia and other genetic diseases by gene therapy.

In November 2006 researchers at the University of Pennsylvania School of Medicine reported on VRX496, a gene-based immunotherapy for the treatment of human immunodeficiency virus (HIV) that uses a lentiviral vector for delivery of an antisense gene against the HIV envelope. In the Phase I trial enrolling five subjects with chronic HIV infection who had failed to respond to at least two antiretroviral regimens, a single intravenous infusion of autologous CD4 T cells genetically modified with VRX496 was safe and well tolerated. All patients had stable or decreased viral load; four of the five patients had stable or increased CD4 T cell counts. In addition, all five patients had stable or increased immune response to HIV antigens and other pathogens. This was the first evaluation of a lentiviral vector administered in U.S. Food and Drug Administration-approved human clinical trials for any disease. Data from an ongoing Phase I/II clinical trial were presented at CROI 2009.

2007

On 1 May 2007 Moorfields Eye Hospital and University College London's Institute of Ophthalmology announced the world's first gene therapy trial for inherited retinal disease. The first operation was carried out on a 23 year-old British male, Robert Johnson, in early 2007. Leber's congenital amaurosis is an inherited blinding disease caused by mutations in the RPE65 gene. The results of the Moorfields/UCL trial were published in New England Journal of Medicine in April 2008. They researched the safety of the subretinal delivery of recombinant adeno associated virus (AAV) carrying RPE65 gene, and found it yielded positive results, with patients having modest increase in vision, and, perhaps more importantly, no apparent side-effects (Maguire *et al*.,2008, Bainbridge *et al.*,2008, Cideciyan *et al*.,2009, Simonelli *et al*.,2010).

2009

In September of 2009, the journal Nature reported that researchers at the University of Washington and University of Florida were able to give trichromatic vision to squirrel monkeys using gene therapy, a hopeful precursor to a treatment for color blindness in humans. In November of 2009, the journal Science reported that researchers succeeded at halting a fatal brain disease, adrenoleukodystrophy, using a vector derived from HIV to deliver the gene for the missing enzyme.

2010

A paper by Komáromy *et al*. published in April 2010, deals with gene therapy for a form of achromatopsia in dogs. Achromatopsia, or complete color blindness, is presented as an ideal model to develop gene therapy directed to cone photoreceptors. Cone function and day vision have been restored for at least 33 months in two young dogs with achromatopsia. However, the therapy was less efficient for older dogs.

2011

In 2007 and 2008, a man being treated by Gero Hütter was cured of HIV by repeated Hematopoietic stem cell transplantation with double-delta-32 mutation which disables the CCR5 receptor; this cure was not completely accepted by the medical community until 2011. This cure required complete ablation of existing bone marrow which is very debilitating.

Vectors of Gene Therapy

Viruses

Viruses have the ability to integrate their genome into that of host cells. Gene therapy exploits this property of virus to us them as vehicle to carry desired gene into the patient’s cell. Following viruses have been used till date for the purpose (http://en.wikipedia.org/wiki/Vectors_in_gene_therapy).

Retroviruses

The genetic material in retroviruses is in the form of RNA molecules. When a retrovirus infects a host cell, it will introduce its RNA together with some enzymes, namely reverse transcriptase and integrase, into the cell. This RNA molecule from the retrovirus produce a DNA copy from its RNA molecule by the enzyme reverse transcriptase before it is integrated into the genetic material of the host cell. The DNA copy is then incorporated into the genome of

the host cell by an enzyme carried in the virus called integrase. Gene therapy trials using retroviral vectors to treat X-linked severe combined immunodeficiency (X-SCID) represent the most successful application of gene therapy to date.

Adenoviruses

Adenoviruses is double-stranded DNA virus. When these viruses infect a host cell, they introduce their DNA molecule into the host. The genetic material of the adenoviruses is not incorporated (transient) into the host cell's genetic material. The DNA molecule is left free in the nucleus of the host cell, and the instructions in this extra DNA molecule are transcribed just like any other gene. The only difference is that these extra genes are not replicated when the cell is about to undergo cell division so the descendants of that cell will not have the extra gene. As a result, treatment with the adenovirus will require readministration in a growing cell population although the absence of integration into the host cell's genome should prevent the type of cancer seen in the SCID trials. This vector system has been promoted for treating cancer and indeed the first gene therapy product to be licensed to treat cancer, Gendicine, is an adenovirus. Gendicine, an adenoviral p53-based gene therapy was approved by the Chinese FDA in 2003 for treatment of head and neck cancer.

Adeno-associated viruses

Adeno-associated viruses, from the parvovirus family, are small viruses with a genome of single stranded DNA. The wild type AAV can insert genetic material at a specific site on chromosome 19 with near 100% certainty. But the recombinant AAV, which does not contain any viral genes and only the therapeutic gene, does not integrate into the genome. Instead the recombinant viral genome fuses at its ends via the ITR (inverted terminal repeats) recombination to form circular, episomal forms which are predicted to be the primary cause of the long term gene expression. There are a few disadvantages to using AAV, including the small amount of DNA it can carry (low capacity) and the difficulty in producing it.

Herpes Simplex Virus

Herpes Simplex Virus is a human neurotropic virus. This is mostly examined for gene transfer in the nervous system. The wild type HSV-1 virus is able to infect neurone. Infected neurones are not rejected by the immune system. Though the latent virus is not transcriptionally apparent, it does possess neurone specific promoters that can continue to function normally. **Non-viral methods**

Non-viral methods present certain advantages over viral methods, with simple large scale production and low host immunogenicity being just two. Previously, low levels of transfection and expression of the gene held non-viral methods at a disadvantage; however, recent advances in vector technology have yielded molecules and techniques with transfection efficiencies similar to those of viruses.

Naked DNA

This is the simplest method of non-viral transfection. Clinical trials carried out of intramuscular injection of a naked DNA plasmid have occurred with some success; however, the expression has been very low in comparison to other methods of transfection. In addition to trials with plasmids, there have been trials with naked PCR product, which have had similar or greater success. This success, however, does not compare to that of the other methods, leading to research into more efficient methods for delivery of the naked DNA such as electroporation, sonoporation, and the use of a "gene gun", which shoots DNA coated gold particles into the cell using high pressure gas or a inverted .22 caliber gun.

Oligonucleotides

The use of synthetic oligonucleotides in gene therapy is to inactivate the genes involved in the disease process. There are several methods by which this is achieved. One strategy uses antisense specific to the target gene to disrupt the transcription of the faulty gene. Another uses small molecules of RNA called siRNA to signal the cell to cleave specific unique sequences in the mRNA transcript of the faulty gene, disrupting translation of the faulty mRNA, and therefore expression of the gene. A further strategy uses double stranded

oligodeoxynucleotides as a decoy for the transcription factors that are required to activate the transcription of the target gene. The transcription factors bind to the decoys instead of the promoter of the faulty gene, which reduces the transcription of the target gene, lowering expression. Additionally, single stranded DNA oligonucleotides have been used to direct a single base change within a mutant gene. The oligonucleotide is designed to anneal with complementarity to the target gene with the exception of a central base, the target base, which serves as the template base for repair. This technique is referred to as oligonucleotide mediated gene repair, targeted gene repair, or targeted nucleotide alteration.

Lipoplexes and polyplexes

To improve the delivery of the new DNA into the cell, the DNA must be protected from damage and its entry into the cell must be facilitated. To this end new molecules, lipoplexes and polyplexes, have been created that have the ability to protect the DNA from undesirable degradation during the transfection process.

Plasmid DNA can be covered with lipids in an organized structure like a micelle or a liposome. When the organized structure is complexed with DNA it is called a lipoplex. There are three types of lipids, anionic (negatively charged), neutral, or cationic (positively charged). Initially, anionic and neutral lipids were used for the construction of lipoplexes for synthetic vectors. However, in spite of the facts that there is little toxicity associated with them, that they are compatible with body fluids and that there was a possibility of adapting them to be tissue specific; they are complicated and time consuming to produce so attention was turned to the cationic versions.

Cationic lipids, due to their positive charge, were first used to condense negatively charged DNA molecules so as to facilitate the encapsulation of DNA into liposomes. Later it was found that the use of cationic lipids significantly enhanced the stability of lipoplexes. Also as a result of their charge, cationic liposomes interact with the cell membrane, endocytosis was widely believed as the major route by which cells uptake lipoplexes. Endosomes are formed as the results of endocytosis, however, if genes can not be released into cytoplasm by breaking the membrane of endosome, they will be sent to lysosomes where all DNA will be destroyed before they could achieve their functions. It was also found that although cationic lipids themselves could condense and encapsulate DNA into liposomes, the transfection efficiency is very low due to the lack of ability in terms of “endosomal escaping”. However, when helper lipids (usually electroneutral lipids, such as DOPE) were added to form lipoplexes, much higher transfection efficiency was observed. Later on, it was figured out that certain lipids have the ability to destabilize endosomal membranes so as to facilitate the escape of DNA from endosome, therefore those lipids are called fusogenic lipids. Although cationic liposomes have been widely used as an alternative for gene delivery vectors, a dose dependent toxicity of cationic lipids were also observed which could limit their therapeutic usages.

The most common use of lipoplexes has been in gene transfer into cancer cells, where the supplied genes have activated tumor suppressor control genes in the cell and decrease the activity of oncogenes. Recent studies have shown lipoplexes to be useful in transfecting respiratory epithelial cells, so they may be used for treatment of genetic respiratory diseases such as cystic fibrosis.

Complexes of polymers with DNA are called polyplexes. Most polyplexes consist of cationic polymers and their production is regulated by ionic interactions. One large difference between the methods of action of polyplexes and lipoplexes is that polyplexes cannot release their DNA load into the cytoplasm, so to this end, co-transfection with endosome-lytic agents (to lyse the endosome that is made during endocytosis, the process by which the polyplex enters the cell) such as inactivated adenovirus must occur. However, this isn't always the case, polymers such as polyethylenimine have their own method of endosome disruption as does chitosan and trimethylchitosan.

Hybrid methods

Due to every method of gene transfer having shortcomings, there have been some hybrid methods developed that combine two or

more techniques. Virosomes are one example; they combine liposomes with an inactivated HIV or influenza virus. This has been shown to have more efficient gene transfer in respiratory epithelial cells than either viral or liposomal methods alone. Other methods involve mixing other viral vectors with cationic lipids or hybridising viruses.

Dendrimers

A dendrimer is a highly branched macromolecule with a spherical shape. The surface of the particle may be functionalized in many ways and many of the properties of the resulting construct are determined by its surface. In particular it is possible to construct a cationic dendrimer, i.e. one with a positive surface charge. When in the presence of genetic material such as DNA or RNA, charge complimentarity leads to a temporary association of the nucleic acid with the cationic dendrimer. On reaching its destination the dendrimer-nucleic acid complex is then taken into the cell via endocytosis.

In recent years the benchmark for transfection agents has been cationic lipids. Limitations of these competing reagents have been reported to include: the lack of ability to transfect a number of cell types, the lack of robust active targeting capabilities, incompatibility with animal models, and toxicity. Dendrimers offer robust covalent construction and extreme control over molecule structure, and therefore size. Together these give compelling advantages compared to existing approaches.

Problems of Gene Therapy:

Some of the problems of gene therapy include (http://learn.genetics.utah.edu/content/tech/genetherapy/gtchallenges/):

Short-lived nature of gene therapy: Before gene therapy can become a permanent cure for any condition, the therapeutic DNA introduced into target cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable. Problems with integrating therapeutic DNA into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving any long-term benefits. Patients will have to undergo multiple rounds of gene therapy.

Immune response: Anytime a foreign object is introduced into human tissues, the immune system has evolved to attack the invader. The risk of stimulating the immune system in a way that reduces gene therapy effectiveness is always a possibility. Furthermore, the immune system's enhanced response to invaders that it has seen before makes it difficult for gene therapy to be repeated in patients.

Problems with viral vectors: Viruses, the carrier of choice in most gene therapy studies, present a variety of potential problems to the patient such as toxicity, immune and inflammatory responses, and gene control and targeting issues. In addition, there is always the fear that the viral vector, once inside the patient, may recover its ability to cause disease.

Multigene disorders: Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some of the most commonly occurring disorders, such as heart disease, high blood pressure, Alzheimer's disease, arthritis, and diabetes, are caused by the combined effects of variations in many genes. Multigene or multifactorial disorders such as these would be especially difficult to treat effectively using gene therapy.

Chance of inducing a tumor (insertional mutagenesis): If the DNA is integrated in the wrong place in the genome, for example in a tumor suppressor gene, it could induce a tumor. This has occurred in clinical trials for X-linked severe combined immunodeficiency (X-SCID) patients, in which hematopoietic stem cells were transduced with a corrective transgene using a retrovirus, and this led to the development of T cell leukemia in 3 of 20 patients.

Future of Gene Therapy

FDA has not yet approved any human gene therapy product for sale. Current gene therapy is experimental and has not proven 100% successful in clinical trials. Presently, gene therapy focuses on treating or curing

existing disease conditions. In the future, the focus should shift to prevention. As more of the human and animal genome is understood, medicine will know more about which genes contribute to or cause disease. With that knowledge in hand, gene therapy could be used to head off problems before their occurrence.

Nonetheless, gene therapy is a very promising approach in the filed of medicine. “Gene therapy will revolutionize medicine over next ten to twenty years, but the big question is….When???”

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