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The Gene Therapy – Exploring the Challenges…

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Abstract: Genes are the smallest functional units of the genetic system, which control the development and function of all organisms. A gene is a linear sequence of DNA that codes for a particular protein. Gene therapy is based on principle that a normal gene is inserted to compensate for a nonfunctional gene and abnormal gene can be repaired through selective reverse mutation. It uses purified preparations of a gene or a fraction of gene to treat diseases. Gene therapy can be used to treat wide range of diseases ranging from single gene disorder to multi-gene disorder. It has variety of applications in the field of dentistry like in cancerous and precancerous condition, salivary gland disorders, autoimmune diseases, bone repair, DNA vaccination, bone repair etc.
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INTRODUCTION

Genes are the smallest functional units of the genetic system, which control the development and function of all organisms. A gene is a linear sequence of DNA that codes for a particular protein [1]. The discovery, six decades ago, of the structure of de-oxy ribonucleic acid (DNA) by James Watson and Francis Crick [28] stands out as the one of the most important findings of the last millennium. DNA is crucial to our existence and small mutations in one or more genetic sequences can have devastating effects for the affected individual.

Gene therapy is based on principle that a normal gene is inserted to compensate for a nonfunctional gene and abnormal gene can be repaired through selective reverse mutation. It uses purified preparations of a gene or a fraction of gene to treat diseases [1]. The gene therapy in essence helps to either replace or inactivate 'bad genes' or introduce new genes for the benefit of the affected individual in order to cure those who suffer from a genetic disease. The recent sequencing of the human genome (The Human Genome Project), now offers us the very real possibility of identifying those genes responsible for a particular genetic disease.

Historical Perspective

Joshua Lederberg and Edward Tatum laid out the fundamental tenets for gene therapy Edward Tatum [2] first envisioned the transfer of therapeutic gene(s), for treatment and cure, two decades earlier than the first gene transfer took place on a clinical setting. The rationale proposed was to treat human diseases emerging from single defect of genes. Gene therapy can be carried out either in vitro (or ex vivo) by treatment of cells or tissues from an affected individual in-culture, with re-introduction into the affected individual, or in vivo if cells cannot be cultured or be replaced in the affected individual.

Gene Therapy by Definition is –

- "Introduction of functional genetic material into target cells to replace / supplement defective genes."
- Gene therapy can be defined as gene transfer for the purpose of treating human disease Cusack and Tanabe, 1998. This includes the transfer of new genetic material as well as the manipulation of existing genetic material.
- Transfer of DNA/RNA to target cells for treatment purposes {nucleic acid based treatment} [3]. (The International Genetic Council; Norway)
- "Human gene therapy is the replacement of person's faulty genetic material with normal genetic material to treat or cure a disease or abnormal medical condition. (U.S. Food and Drug Administration) [4].

Thus, Gene therapy is the transfer of new genetic material into a cell for therapeutic benefit. This can be accomplished by: 1) replacing or inactivating a dysfunctional gene, 2) replacing or adding a functional gene, or 3) inserting a gene into a cell to make it function normally.

Early Trends

In 1970s, scientists proposed "gene surgery"

for treating inherited diseases caused by faulty genes. The idea was to take out the disease-causing gene and surgically implant a gene that functioned properly [5, 6].

During 1980s, gene therapy gained an established foothold in the minds of medical scientists as a promising approach to treatments for specific diseases. One of the major reasons for the growth of gene therapy was scientists' increasing ability to identify the specific genetic malfunctions that caused inherited diseases.

In 1990, on September 14th, a four-year old girl suffering from a genetic disorder that prevented her body from producing a crucial enzyme, ADA (adenine deaminase)/SCID became the first person to undergo gene therapy in the United States. She had a weakened immune system, making her extremely susceptible to severe, life-threatening infections. W. French Anderson and colleagues at National Institutes of Health's Clinical Center in Bethesda, Maryland, took white blood cells (which are crucial to proper immune system functioning) from the girl, inserted ADA producing genes into white blood cells, and then transfused the cells back into the patient. Debate arose as to whether the improvement resulted from the gene therapy or from an additional drug treatment she received [7-9].

Sequence and Consequence

A successful gene function requires coding regions and regulatory elements to be present and to be in the correct alignment. The delivery of exogenous DNA and its processing by target cells requires the introduction of new pharmacological paradigms. The end result should take into account the fate of altered gene expression and protein function. Ledley and Ledley in 1994 have described a multi compartmental model to describe the events involved in the gene transfer in a quantitative fashion. The gene transfer is carried by use of various biological/ physical/ chemical delivery methods known as vectors [10].

Types of Gene Therapy

Somatic gene therapy

Somatic gene therapy is limited to the individual undergoing treatment. Somatic gene therapy involves the manipulation of gene expression in cells that will be corrective to the patient but not inherited to the next generation. Somatic cell gene therapy is at an early stage of development. Somatic gene therapy may be divided into three categories: [11-13].

A. Ex – vivo

It involves removing cells from the body and treating them with the vector carrying the therapeutic DNA and then returning the genetically modified cells to the organism. So far this has mostly been an option with blood cells and skin cells.

B. In –situ

Placing the vector carrying the therapeutic DNA directly into the tissue in which the gene will function. This has been tried out for airways/ lungs in cystic fibrosis and some forms of cancer.

C. In vivo

Here the therapeutic DNA – carrying vector might for instance be injected into the blood stream to be taken up by target cells. (Fig. 1)

Germ line based gene therapy

Germ line based gene therapy will cause a genetic change that will be inherited. Germ line gene therapy involves the genetic modification of germ cells (sperms and eggs) in order to prevent a genetic defect from being transmitted to future generations. (Fig.2)

Types of Gene Therapy Methods

Gene therapy is divided into 4 levels by the geneticists-

- Gene transfer with integration (gene incorporated into the DNA)
- Gene transfer without integration (gene not incorporated into the DNA)
- The use of small synthetic oligonucleotides, so called ribozymes / antisense molecules without regulatory elements which modify gene expression.
- Therapeutic DNA vaccines.

Monogenic hereditary diseases, primary immuno-deficiencies and cystic fibrosis are important groups, but gene therapy today also encompasses other disease groups e.g. - pre-cancerous, cancerous, infectious diseases {mainly HIV} and others. In the early 1980's, gene therapy was introduced in the health services abroad, with the promises of a new age in medicine with potential therapies for all serious diseases. Preliminary trials indicate that gene therapy is a safe method with surprisingly few, mostly local side effects.

The most important barriers in today's gene therapy are the problem of *gene transfer*. The main features being, more efficient and targeted gene transfer and controlled gene expression in the targeted cell.

Gene Transfer

Gene therapy potentially represents one of the most important developments to occur in medicine, but before this can be realized certain technical problems common to all methods of gene delivery must be overcome. In order to modify a specific cell type or tissue, the therapeutic gene must be efficiently delivered to the cell, in such a way that the gene can be expressed at the appropriate level & for a sufficient duration. Two broad approaches have been used to deliver DNA to cells, namely viral vectors & non-viral vectors, which have different advantages as regards efficiency, ease of

production & safety.

In gene therapy the gene or the gene fragment that is of interest is usually inserted in a vector (often modified virus DNA/RNA or plasmid DNA) that is a crucial part of the transfer system (transport vehicles). Therapeutic DNA is inserted in the vector at a place where it often replaces DNA that is not essential for the transfer or multiplication of virus. Work is in progress on a range of different transfer systems.

Gene Delivery Systems

Genetic material can be transferred *via* a vector that is defined as the vehicle that is used to deliver the gene of interest. The ideal vector would transfer a precise amount of genetic material into each target cell, thereby allowing for expression of the gene product without causing toxicity. Chemical transfection introduces DNA by calcium phosphate, lipid, or protein complexes. Lipid vectors are generated by a combination of plasmid DNA and a lipid solution that result in the formation of a liposome [14]. This fuses with the cell membranes of a variety of cell types, introducing the plasmid DNA into the cytoplasm and nucleus, where it is transiently expressed. Many carcinoma cells, including oral squamous cancer cells, express high levels of folate receptor. Linkage of DNA or DNA-lipid complexes to folate can specifically target cancer cells. Pre-clinical studies have demonstrated the potential utility of linking targeting moieties to the gene therapy construct. The DNA can then be internalized *via* receptor-mediated endocytosis. (Fig.3)

Physical transfection of genes can be accomplished by electroporation, microinjection, or use of ballistic particles. Electroporation therapy with intralesional bleomycin has been reported to be a technically simple outpatient technique where high-voltage electric impulses can be delivered into a neoplasm by transiently increasing cell membrane permeability to large molecules, including cytotoxic agents, thus causing localized progressive necrosis. Unlike many laser ablation methods, electroporation can treat bulky tumors (> 2 cm) with complete penetration [12]. Clinical trials demonstrated that electroporation was safe and efficacious in 14 patients with squamous cell carcinoma. Particle bombardment has been studied to deliver genes to the oral mucosa in preclinical animal models. Although gene transfer to mucosa has shown anti-tumor effects, the limited transfection efficiency must be addressed prior to clinical application.

Virus based vectors

Retrovirus (RNA virus), including lentivirus, Adenovirus (DNA virus), Adeno-associated virus (DNA-virus), Herpes simplex virus (DNA virus)

Vaccinia virus (DNA virus) Plasmid DNA, Liposome bound DNA, Protein DNA conjugates, artificial chromosomes.
(Fig. 4), Table 1

DNA and RNA Molecules

DNA is the most important substance known to humanity since it carries within its structure the hereditary information that determines the structures of the proteins – the essential elements that are the building blocks for all cells and tissues. DNA also provides the instructions for directing cells to grow and divide and sends the messages required by fertilized eggs to differentiate into the multitude of specialized cells that make up our bodies [11]. Since Watson and Crick first published their double helical model of DNA in 1953, investigators have begun to understand more clearly how DNA controls the expression of the genes and proteins within the cells and consequently why one particular cell differentiates into a completely different cell type e.g. liver cell.

Chromosomes and Chromatin

DNA is packaged within chromosomes in the nuclei of cells. Chromosomes are relatively large particles (a few micrometers) that are visible by light microscopy. Each chromosome is composed of a centromere from which protrude four arms, each sealed by telomere that helps to confer stability to the ends of the chromosome. Essentially, these structures can be regarded as assemblies of units made up of DNA, RNA and proteins, which are precisely duplicated during each cell division. Chromatin is composed of a string of DNA, approximately 1metre long and 0.2nm wide, wound around a core of proteins called histones. Chromatin itself is composed of individually packed units called nucleosomes, which, by electron microscopy, appear as beads on a thin string.

When chromosomes become abnormal, for eg. due to the effects of certain drugs, radiation or other noxious agents, they can lead to the development of cancer or related diseases. Such chromosomal abnormalities can be detected by molecular biological (capacity to precisely study and manipulate nucleic acids) [15] analyses such as amounts of DNA (in the form of complementary DNA or c DNA) into larger quantities that can be used for analysis. Indeed, approaches such as PCR are used routinely in the diagnosis of such genetic disorders.

Use of DNA as A Drug

A number of human diseases are known to be genetic in origin (e.g. Huntington's chorea, cystic fibrosis) and virtually all diseases, except for some trauma, have a hereditary component. Thus, the opportunity to treat such disorders by replacing the defective gene(s) with a normal healthy gene (gene therapy) offers a novel therapeutic approach for patients who suffer from such diseases

The sequencing of the human genome will permit the relatively simple identification of genes associated with a particular disease and also pin point exactly that where on the three million base pair DNA strand the gene is located. Such genetic profiling will also enable the physicians to predict which drugs will and will not work for a particular patient. Indeed, this new field of medicine, called Pharmacogenetics [16,17], will enable a doctor to tailor therapy to an individual's requirements. Despite these ideals, there still is a long way to go before such technology can become routine. One reason for this is that, unlike monogenetic disorders such as severe combined deficiency syndrome (SCID), which is caused by the mutation in the adenosine de-aminase (ADA) gene, very few diseases are caused by a single gene mutation; most are caused by the mutation of multiple genetic components. For eg. Cancer usually involves multiple genetic lesions within the same cell and it is unlikely that the nature of everyone of these oncogenic mutations are yet known. Thus, in its broadest terms, gene therapy represents '*an opportunity for the treatment of genetic disorders in adults and children by genetic modification of human body cells*' (Report of the UK Health Minister's Gene Therapy Advisory Committee, 1995).

Although gene therapy is a simple concept, in practice it poses a number of complications. For instance, the correct gene must be inserted into the correct cells and expressed in those cells at the correct time. Also, gene expression normally should be maintained for long periods (for the lifespan of the patient in the case of inherited diseases) in order to minimize the number of times a patient requires treatment. Overcoming such problems for each disease is a formidable task and, despite the early promise of this form of treatment, this probably accounts for the limited clinical success that has been achieved to date with gene therapy.

Several general strategies utilized in a gene therapy approach to cancer, including:

- Addition of a tumor-suppressor gene (gene addition therapy);
- Deletion of a defective tumor gene (gene excision therapy);
- Down-regulation of the expression of genes that stimulate tumor growth (antisense RNA);
- Enhancement of immune surveillance (immunotherapy);
- Activation of prodrugs that have a chemotherapeutic effect ("suicide" gene therapy);
- Delivery of drug resistance gene(s) to normal tissue for protection from chemotherapy; and
- Introduction of genes to inhibit tumor angiogenesis.

All of the gene therapy trials currently approved for the use in humans targets somatic cells that will live only as long as the patient. This ensures that the genetic treatment will affect only one generation and will not alter the genetic makeup of any offspring of the patient, assuming there is no inadvertent spread of the therapeutic gene(s) to the gametes. This is known as Somatic gene therapy, and its purpose is to alleviate disease in the treated individual, and in that individual alone. [4]In contrast, it also is possible to target directly the gametes (sperm and ova) in order to modify the genetic profile, not of the current, but of the subsequent generation of unborn 'patients'. Gene transfer at an early stage of embryonic development also might have similar effects by achieving gene transfer to both somatic and germ line cells. This is Germ line gene therapy. The attraction of germ line gene therapy for the treatment of disease is that, at least in theory, permanent genetic cures might be achieved by delivering a functional copy of a mutated gene to every cell of the resulting progeny. However, there is extensive apprehension about the development of germ line gene therapy research programmes. The ability to alter the genetic profile of subsequent generations rightly invokes many spectres. Apart from the inability to predict the long-term sequelae of altering the germ line by delivery of the exogenous genetic material at the scientific level, there are many ethical issues raised by the prospect of treating 'patients' whose consent it is impossible to obtain. In addition, although currently it is not possible to manipulate genetically traits such as 'intelligence' or 'beauty', there is a perceived fear of such technologies being abused in eugenic-type breeding programmes in the future? As a result, the major ethical and regulatory bodies of gene therapy both in the US and Europe have placed a moratorium on the consideration of any germ line gene therapy treatments of human patients because of 'insufficient knowledge to evaluate the risks to future generations'.

Gene Therapy Strategies for Oral Cancer

Oral cancer is a particularly appropriate target for gene therapy, since direct injection of genes into most primary and recurrent lesions is possible. The gene therapy approach is also amenable to cancer cell-specific gene delivery and expression, which will alleviate the problem of destroying normal cells during therapy [18].

Gene therapies that result in cancer cell death include:

- Gene addition therapy
- Gene excision therapy [19]
- Antisense ribonucleic acid (RNA) therapy
- RNA interference (RNAi)-based gene therapy
- Immunotherapy
- Suicide gene therapy
- Gene therapy with the use of oncolytic viruses
- The delivery of drug resistance genes into normal tissues for protection against chemotherapy [20].

Gene Addition Therapy

Normal cells have the ability to regulate the cell cycle and eventually undergo programmed cell death (apoptosis). Cancer cells generally demonstrate impaired cell-cycle progression, largely due to mutations and overexpression of cell-cycle regulators. Several genetic alterations have been described in oral cancer, including mutations of p53, the retinoblastoma gene (RB1), p16, and p21. The most extensively studied mutations in oral cancer are those of p53. Since the protein p53 plays a role in cell-cycle regulation and in apoptosis, p53 gene transfer was initially tested in squamous cell carcinoma patients by injecting the primary or regional tumor with an adenoviral vector expressing wild-type p53. Adenoviral p53 (Ad-p53) was demonstrated to be safe and well tolerated [21, 22].

Antisense RNA and Ribozymes

RNA that is complementary to the strand of DNA expressing the gene can usually inhibit Gene expression. This "antisense" RNA can prevent the activity of several known oncogenes, including myc, fos, and ras, and can inhibit viruses such as HSV-1, HPV, and HTLV-1. Such therapy can theoretically be directed toward carcinoma cells whose malignant phenotype is dependent upon the expression of particular oncogenes. Inhibition of expression of these oncogenes may alter the phenotype, thus abrogating tumor growth.

Suicide gene therapy

This therapy involves enzymes, the expression of which transforms the non-toxic producing drug into an active cytotoxic substance. It is the most commonly used gene therapy and uses thymidine kinase or other chemosensitizing genes [23]. Viral Herpes simplex thymidine kinase (HSVtk) converts a nontoxic prodrug ganciclovir (GCV) into a toxic form thereby killing the cells expressing the enzyme [24].

THE HUMAN GENOME

A genome is the entire DNA in an organism, including its genes. The human genome is made up of 23 chromosome pairs with a total of 2.9 billion base pairs encoding approximately 20,000-35,000 genes. Genomes vary widely in size: the smallest known genome for a free-living organism (a bacterium) contains about 600,000 DNA base pairs, while human and mouse genomes have some 3 billion DNA base pairs. Except for mature red blood cells, all human cells contain a complete genome [25].

The Human Genome Project (HGP) was the international, collaborative research program whose goal was the complete mapping and understanding of all the genes of human beings. The HGP was the natural culmination of the history of genetics research. In 1911, Alfred Sturtevant, then an undergraduate researcher in the laboratory of Thomas Hunt Morgan, realized that he could - and had to, in order to manage his data - map the locations of the fruit fly (*Drosophila melanogaster*) genes whose mutations the Morgan laboratory was tracking over generations [25].

Begun formally in 1990, the U.S. Human Genome Project was a 13-year effort coordinated by the U.S. Department of Energy and the National Institutes of Health. The project originally was planned to last 15 years, but rapid technological advances accelerated the completion date to 2003.

Project goals were to identify all the approximately 20,000-25,000 genes in human DNA,

- Determine the sequences of the 3 billion chemical base pairs that make up human DNA,
- Store this information in databases,
- Improve tools for data analysis,
- Transfer related technologies to the private sector, and
- Address the ethical, legal, and social issues (ELSI) that may arise from the project.

To help achieve these goals, researchers also studied the genetic makeup of several nonhuman organisms. These include the common human gut bacterium *Escherichia coli*, the fruit fly, and the laboratory mouse [26]. The HGP has revealed that there are probably somewhere between 30,000 and 40,000 human genes. The completed human sequence can now identify their locations. This ultimate product of the HGP has given the world a resource of detailed information about the structure, organization and function of the complete set of human genes. This information can be thought of as the basic set of inheritable "instructions" for the development and function of a human being.

The International Human Genome Sequencing Consortium published the first draft of the human genome in the journal *Nature* in February 2001 with the sequence of the entire genome's three billion base pairs some 90 percent complete. A startling finding of this first draft was that the number of human genes appeared to be significantly fewer than previous estimates, which ranged from 50,000 genes to as many as 140, 000. The full sequence was completed and published in April 2003.

Hazards of Gene Therapy

Probably the biggest setback for the gene therapy occurred recently in the autumn of 1999 when Jesse Gelsinger, an 18–year–old high-school graduate from Arizona, died as a result of gene therapy experiment. Gelsinger developed a fever and blood clots throughout his body within hours of treatment to correct partial ornithine transcarbamylase (OTC) deficiency, a rare metabolic disease that can cause a dangerous build up of ammonia in the body. He died

four days later. Although the exact reasons for the failure of the gene therapy remain unclear, researchers are investigating the adenovirus vector used to deliver the OTC gene to the liver. Although Gelsinger was 18th and the final patient in the phase I experiment, he was only the second person to receive a dose of 3.8×10^{13} virus particles, believed to be the highest so far with an adenovirus. As a direct consequence of that incident, the University of Pennsylvania Institute for Human Gene Therapy in the USA had its clinical trials programme terminated [27].

CONCLUSION

Change and modification is a continuous process in advancement of technology. Research is being done to understand the cellular and molecular basis of every disease. Most of the conventional methods to treat diseases have not been giving satisfactory results, so currently there is increasing focus on gene therapy to treat wide variety of inherited and acquired diseases. Gene therapy can be used to treat wide range of diseases ranging from single gene disorder to multi-gene disorder. It has variety of applications in the field of dentistry like in cancerous and precancerous condition, salivary gland disorders, autoimmune diseases, bone repair, DNA vaccination, bone repair etc. As investigations into the genomics and unique molec- ular architecture of cancers continue new therapeutic tar- gets will no doubt be revealed. Biomarkers present in saliva have already revealed a number of new genetic and epigenetic targets. New targeting agents such as broad-spectrum kinase inhibitors show great promise in clinical trials.

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