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RESEARCH ARTICLE

AN INSIGHT INTO GENE THERAPY - THE FOREFRONT OF MEDICINE

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ABSTRACT

Gene therapy focuses at introduction of a defined DNA sequence into particular cells of a patient either to substitute a faulty gene, or to accord a new function to the cell for secretion of protein with alleged therapeutic function. The term 'gene transfer' refers to the delivery of a gene, a cDNA, a small RNA, that is, any type of oligonucleotide that might have some therapeutic benefit, to a predetermined target cell. Gene therapy allows specific gene products to be delivered to a precise anatomic location. In addition, the level of transgene expression as well as the duration of expression can be regulated with current techniques. The purpose of this review is to give a brief insight into the gene transfer principles, mechanisms and strategies for correcting various diseases.

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INTRODUCTION

"We used to think that our fate was in our stars, but now we know that, in large measures, our fate is in our genes," quoted James Watson. The fate Watson talks about is in our genes, in accordance with the upcoming technique, gene therapy. Gene therapy focuses at introduction of a defined DNA sequence into particular cells of a patient either to substitute a faulty gene, or to accord a new function to the cell for secretion of protein with alleged therapeutic function. The term 'gene transfer' refers to the delivery of a gene, a cDNA, a small RNA, that is, any type of oligonucleotide that might have some therapeutic benefit, to a predetermined target cell. Gene transfer can be considered permanent, for example, with the transferred gene integrating into a chromosome of the targeted cells, or transient, that is, the transferred gene being located extrachromosomally. Gene transfer can be an isolated therapy, used alone, or it can be adjunctive, used in combination with other, more conventional therapies, most notably in cancer treatments. (BJ Baum, 2014)

The underlying principles of gene therapy include

- Insertion of a normal gene into a nonspecific site within the genome to replace an abnormal gene;
- Homologous recombination wherein a normal gene swaps an abnormal gene
- Repair of the abnormal gene through selective reverse mutation, which returns the normal functions of gene
- The regulatory switching of a specific gene could be managed. (Mahale *et al*., 2009)

A limelight into basics of genetics

Diseases in humans can occur due to a multitude of genetic and environmental factors which act in sync with each other. Genetic factors predominate in certain conditions such as Down syndrome, while environmental factors predominate in diseases such as tuberculosis. Most chronic non-communicable conditions such as schizophrenia and diabetes are caused by an interaction of both genetic and environmental factors as depicted in Fig. 1. Genes are made of deoxyribonucleic acid (DNA) and carry our traits through generations. They serve as guiding tomes for production of functional molecules such as ribonucleic acid (RNA) and proteins. A mutation is defined as an alteration in the genetic material through exposure to mutagenic agents, or abruptly through defects in DNA replication and repair. Mutations can be somatic that leads to adult-onset disease such as cancer and cannot be passed on to future generations. Conversely, mutations in gametes or gonadal tissue can be transmitted to future generations. (http://www.gfmer.ch/SRH-Course-2010/course-files/pdf/Introduction-basic-human-genetics-Hamamy-2010.pdf)

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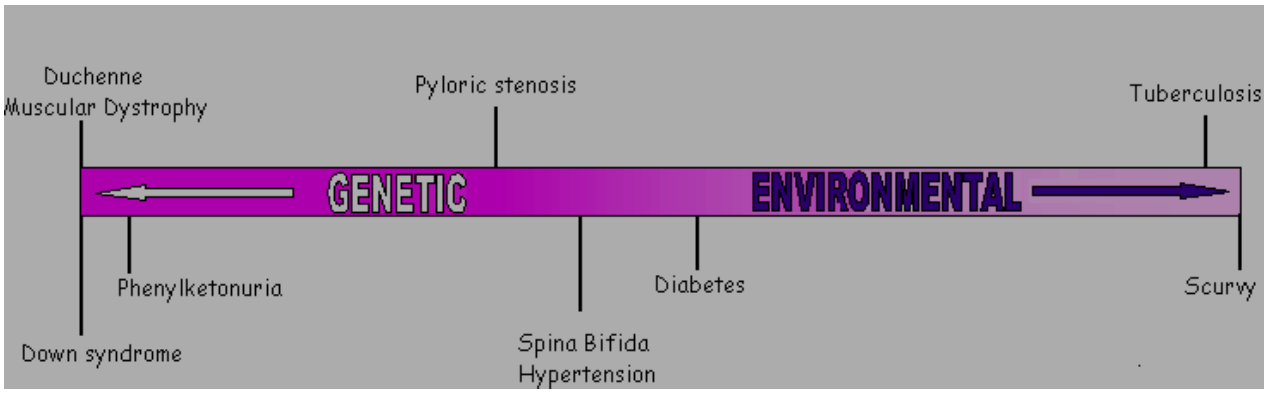

Fig. 1. A schematic representation of genetic and environmental influences on diseases

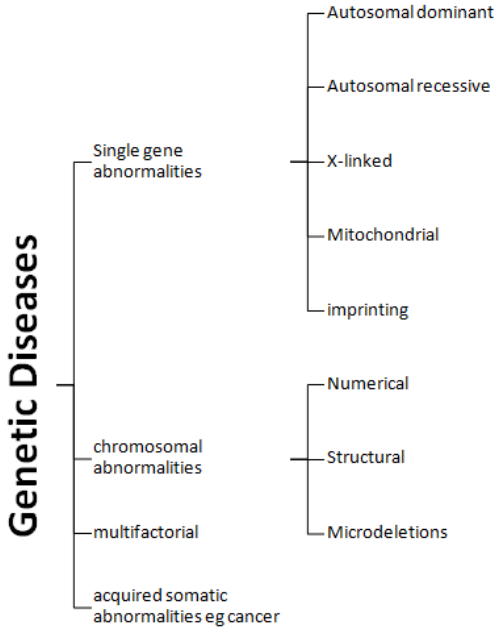

Fig. 2. Broad classification of genetic diseases

A broad classification of genetic diseases is given in Table 1.

The historical perspective

Austrian monk Gregor Mendel, in 1850s, began the scientific study of genetics which laid the foundation for further advancements that presaged the era of modern genetics. With the advent of genetic expansions, the platform was set for genetic engineering, which has produced new drugs and antibodies and enabled scientists to contemplate gene therapy. Fig 2 gives a brief insight over the timeline of genetics. (Vries, H. de, 1889; Ernest *et al*., 2002; William Bateson's letter to Adam Sedgwick, Bateson and William 1907; Online summary of "Real Genetic vs. Lysenko Controversy, Beadle and Tatum, 1941; Oswald *et al*., 1944; Luria, 1947; Bernstein, 1981; Hershey and Chase, 1952; Watson and Crick, 1953; Wright, Pearce, 2001; Meselson and Stahl, 1958; Jacob *et al*., 1960; Jacob, 1960; Crick *et al*.,1961; Min Jou *et al*., 1972; Fiers *et al*., 1976) Joshua Lederberg wrote in 1963: "We might anticipate the in vitro culture of germ cells and such manipulations as 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" Almost after 25 years, in 1989, the first clinical human trial was performed by Rosenberg *et al* performed and neomycin resistance marker gene was transferred into tumor-infiltrating lymphocytes acquired from patients having metastatic melanoma. With this study, a sparkling and hopeful foundation was laid for the treatment of diseases without conventional cure. (Lederberg, 1963)

From 1990 till 1999, the number of clinical trials escalated rapidly. But in 1999, Jesse Gelsinger, an 18-year old suffering from ornithine transcarbamylase deficiency and participating in a gene therapy trial died due to an unexpected calamitous inflammatory reaction to the adenoviral vector. Therefore, in January 2000, trials were put on hold by the US Food and Drug Administration. However soon after in 2000, hopes were raised after successful treatment of children suffering from severe combined immunodeficiency (SCID-X1).

1865 - Gregor Mendel studied the inheritance of traits by experiments on garden pea plants and formulated the basic principles of inheritance, namely, the principles of independent assortment, and segregation

1869 - J.F Miescher isolated cell nuclei,. now known as nucleic acids

1889 - Hugo de Vries postulates that "inheritance of specific traits in organisms comes in particles", naming such particles "(pan)geneVries

1902 - Archibald Garrod discovered inborn errors of metabolism. He deduced that Alkaptonuria was prevalent in populations whose parents had consanguineous marriages.

1904 - Walter Sutton and Theodor Boveri gave the chromosome theory which was concluded to be the basis of the physical basis of the Mendelian law of heredity.

1910 – Thomas Morgan established the nature of sex-linked traits which also asserted the Chromosome Theory of Heredity

1923 – Frederick Griffith studied the transformation of bacteria and discerned that the pathogenic genes are carried by DNA

1931: Crossing over is identified as the cause of recombination

1933 - Thomas Morgan determined the role played by the chromosome in heredity by Linkage Mapping.

1941 - Edward Lawrie Tatum and George Wells Beadle show that genes code for proteins;

1950 – Erwin Chargaff established the pairing method for nitrogenous bases and formulated the Chargaff/s rules.

1953 - James Watson and Francis Crick proposed a double helix model for the structure of DNA.

1970: Restriction enzymes were discovered in studies of a bacterium, *Haemophilus influenzae*, enabling scientists to cut and paste DNA

1972-73 The first publications describing the successful production and intracellular replication of recombinant DNA appeared - inventors as Stanley N. Cohen and Herbert W. Boyer

1976 - Frederick Sanger and Charles Coulson devised the method of DNA sequencing.

1985 - Alec Jeffreys announced DNA fingerprinting method.

1997 - Ian Wilmut cloned Dolly the Ship.

2003 – The Human Genome Project is complete.

(Raper *et al*., 2003) With this, a debate on the risk-benefit ratio of Gene Therapy initiated and the joint statement of the American Society of Gene Therapy (ASGT) and the European Society of Gene Therapy (ESGT) clearly summarises the current view which was published in response to an exceedingly negative article in Nature. The two Societies stated: ‘‘The field of genetherapy is working to develop new and better methods totreat a variety of severe disorders, including genetic diseasessuch as hemophilia and SCID, and also cancer and AIDS. The clear-cut therapeutic benefits seen in recent clinicaltrials of gene therapy for XSCID and ADA-deficient SCIDwarrant judicious consideration of the benefits and risks of this approach compared to imperfect alternatives, such ashaplo-identical hematopoietic stem cell transplantation.” (http://www.wiley.co.uk/genmed/clinical)

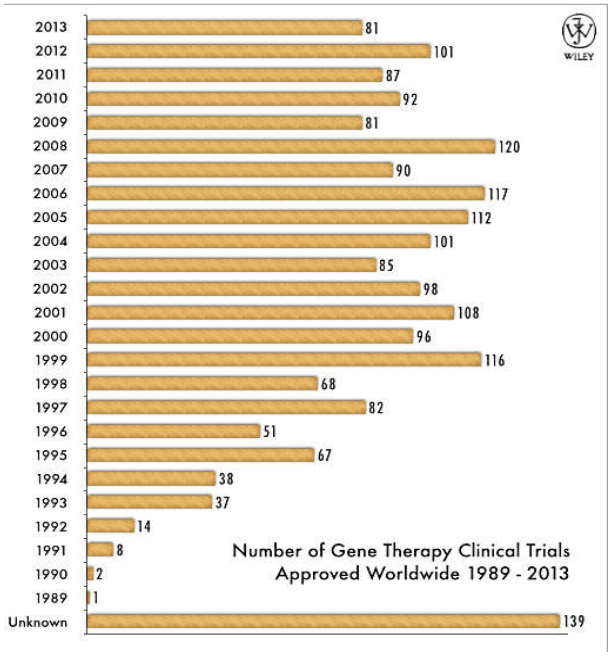

Fig. 3. Statistical analysis of the number of gene therapy trials approved in 1989-2013

Approaches of gene therapy

2 Basic approaches

1. Somatic

In somatic gene therapy, the therapeutic genes are transmitted to the somatic cells, or body, of a patient. Therefore the effects and alterations are not passed on to off springs but is limited to the individual. Somatic gene therapy is the mainstay of present clinical research, wherein the therapeutic DNA transgene (as plasmid or integrated in the genome) is used. (Misra, 2013)

2.Germ-line

In germline gene therapy, functional genes are transmitted to the germ cells (sperm or eggs) of the patient wherein it gets incorporated into the genome. This makes the therapy inheritable and transmitted to off springs. Although theoretically this is exceedingly effective in countering genetic disorders and hereditary diseases, some jurisdictions for bidhuman trials, at least presently, for ethical and methodical reasons, including scarcedata about likely jeopardies to future generations and greater safety concerns in comparison to somatic gene therapy. (Misra, 2013)

Gene delivery

1. Ex vivo - Cells are removed from body and incubated with vector; engineered cells are then returned
2. In situ - Vector or producer cells are placed directly into the tissues to be transduced
3. In vivo - Vector would be directly injected into the bloodstream andwould home in on its target cells. (Ana, 2008)

Vectors (Michael *et al*., 2004; Anderson, 1998; Templeton *et al*., 2008)

1.Viral vectors

Innocuous viruses, because of their naturally evolved property of encapsulating and proficiently relocating their genes into host cells, have become the mostlooked-forvectors for therapeutic gene delivery. The viral vectors currently under trials are based on both DNA and RNA viruses processing varied genomic structures and host ranges. Some viruses insert their genes into the host genome without essentially entering the cell while others breach the cell membrane masquerading as a protein molecule and enter the cell. After being transmitted to the right location, once the transplanted gene is ‘switched on’, it can signal necessary instructions for the production of protein, which was previously missed or altered. In almost 70% of clinical gene therapy trials worldwide, retroviruses, adenovirus, adeno-associated virus, herpesvirus are the viral vectors under study. Amongst all the vector systems, retrovirus vectors signifies the most conspicuous delivery system, since they have high genetransfer efficiency and facilitate high expression of therapeutic genes. DNA viruses such as adenovirus-, adeno-associated virus or herpesvirus are also very efficient for gene delivery and thenumber of clinical trials using these vectors is rapidly growing.

Non-viral vectors

The goal of developing non-viral vectors is to design a system that is highly efficient with a sustained gene expression and low toxicity. Nonviral vectors have an edge over viral vectors because of the ease and simplicity of use, large-scale production is easy and they do not initiate a specific immune response.

These techniques are broadly divided into two general categories:

(1) A physical method for the naked DNA delivery, such as electroporation and gene gun, and
(2) Throughchemical carriers such as cationic polymer and lipid.

Virus	Advantages	Disadvantages
Retrovirus (such as Murine Leukemia Virus-MuLV)	-Well known, easily managed -Includes up to 9 kb of exogenous genetic material -None of the viral proteins or de novo synthesis of viral proteins is required for viral expression. -The 3 basic components of viral particles can be viewed as discrete components and 'mix-matched' for custom applications. -Efficient transfer and high levels of expression -Permanent expression and passed on to daughter cells by viral integration in genome through integrase protein.	-Infects only dividing cells, hence transduction efficacy is low -Chances of insertion mutagenesis -Capacity for insertion of foreign sequences is low. -Low titres -Possible generation of infective viruses -Instability of the engineered vectors
LENTIVIRUS (such as HIV-1)	-Able to infect nondividing cells, useful for targeting well-differentiated, cell, such as in epithelial tissues.	-Risk of RCR (replication-competent retrovirus) arising by recombination
ADENOVIRUS	-Ad are not oncogenic in humans, -the genomes of common Ad are completely defined, -Ad genome can be easily modified, and recombinant -Highly concentrated without modifying the ability of the virus to infect cells. -Infect both dividing and non-dividing cells; -transduction efficacy is high -Includes up to 7.5 kb of exogenous genetic material -High titres -Insertion mutations are avoided as it does not integrate with the genome	-Expression is transitory in nature, declines in 2-4 weeks; thereby requiring periodic treatments -Lower expression levels -Possible immune and inflammatory reactions -Risk of multiplication
ADENO-ASSOCIATED VIRUS	-Parental viruses do not cause disease. -Smallest and most chemically defined particulate gene delivery system -No undesirable cellular immune responses and appear not to induce inflammatory responses -The vectors readily transduce dividing or nondividing cells -can persist essentially for the lifetime of the cell	-Limited DNA payload capacity of about 4.5 kilobases (kb) per particle. -Lacks tissue specificity -
HERPES VIRUS	-Thymidine kinase expression; high efficacy of gene transfer -large viruses, with the prospective to house multiple transgene cassettes -mechanisms that allow lifelong persistence in a non-integrated latent state -does not require cell division for infection and gene expression -Capable of distributing genes to pluripotent cells and their differentiated progeny -may be most suited for expression of genes in the nervous system where the virus has evolved to remain latent.	-Gamma-herpes viruses are sometimes associated with malignancy
VACCINIA	-wide host range -genome has been completely sequenced, facilitating the creation of recombinant vectors. -It can hold up to 25 kb of foreign DNA without a need for viral deletions -cell infected with the virus is rapidly killed, and cell-to-cell spread is efficient -Virus is stable, integration into the genome is very unlikely.	-Long term gene expression not feasible -Chances if viremia in immunocompromised host -As vaccinia virus injections led to scarification of skin, scarification of other organs can lead to poor outcomes.
BACULOVIRUS	-High capacity of incorporation of foreign DNA -Safe, No toxicity, allergic responses, or pathogenicity associated with the baculoviruses -Easy production	-efficiently destroyed by complement
BACTERIA AS VECTORS	-plasmids of almost unlimited size, -plasmid-carrying microbes can be produced and stored at low cost -bacterial vectors can be applied mucosally via their natural route of infection -metabolically attenuated vaccine strains are available for many approved bacterial species -bacterial infection can be managed by antibiotics	-virulence factors released from the bacteria can have severe toxic effects on some cells -unexplained tolerance induction in some cases -additional cytokine therapy might be needed -

Naked DNA delivery

Systemic injection of the naked plasmid DNA into tissue or into a vessel is the simplest way of naked DNA delivery. According to different studies, skeletal muscle, cardiac muscle, liver, thyroid, urological organs, skin and tumor are by far the safest sites for naked DNA delivery. The use of systemic injection for gene administration is limited because of fast degradation by serum nucleases and its clearance through mononuclear phagocyte system, the level of expression and limited area after naked DNA injection. Improved efficiency is achieved through various physical manipulationslike electroporation, bioballistic (gene gun), ultrasound and hydrodynamics (high pressure) injection. Electroporation facilitates permeabilization of cells through the application of controlled electric fields. For the augmentation of gene uptake into cells after the injection of naked DNA, electroporation is used. Additionaly, electroporation ensures long-lasting expressions and it can be used in several tissues. Skin is one of the ideal target sites owing to the accessibility and ease of administration. Gene gun or a bioballisticparticle delivery system is a device for delivering plasmid DNA coated with an elemental particle of a heavy metal directly into tissues or cells. It allows the direct penetration of gene into the cytoplasm or nucleus through the cell membrane, bypassing the endosomal compartment. Recently, studies to introduce genes for antigen or cytokines such as IL-12 into skinor liver for vaccination and immunotherapy have been done. However, shallow tissue penetration of DNA is a major disadvantage of this method. Ultrasound increases the cell membrane permeability to macromolecules such as plasmid DNA is also known to enhance the gene expression when ultrasonic wave is irradiated to the tissue after DNA injection. The use of ultrasound is safe and flexible, and its use with microbubble further enhances levels of gene expression. Microbubbles (ultrasound contrast agents) lower the threshold of cavitation produced by ultrasound energy. Perfluoropropanefilled albumin microbubbles are commonly being used in recently reported studies. Before injecting, it is modified with plasmid DNA and then irradiated with ultrasound. Currently, this technique is being utilised for gene delivery into muscle and vascular cells. Hydrodynamic injection is a technique in which solution of naked DNA is injected in a large volume. It has been hypothesised that a large volume of solution is accumulated in the liver through a receptor-mediated pathway by hepatocytes and the flexible structure of liver. Furthermore, DNA is forced into the liver cells before it is mixed with blood and breaks the endothelial barrier which results in a highly efficient expression in the liver.

Gene delivery using a chemical carrier

Gene carriers can be categorized into several groups

1. Carriers that form complex with DNA and protect DNA from blood components like nucleases
2. Carriers that deliver genes to specific cells
3. Carriers that increase DNA delivery to the nucleus or cytosol
4. Carriers designed such that they dissociate from DNA in the cytosol
5. Carriers designed to deliver a continuous expression of gene into the tissue.

Chemical carriers commonly in use are cationic lipids, polymers or proteins that condense DNA into particles of size 100 to 300 nm by forming a complex with it.

Lipid-mediated gene delivery

Felgner in 1987, first reported Liposome-based gene delivery. Numerous cationic lipids have been reported, such as cationic derivatives of cholesterol, quaternary ammonium detergents, and lipid derivatives of polyamines. Cationic liposome–nucleic acid complexes can be administered via numerous delivery routes in vivo including direct injection (e.g., intratumoral), intravenous, intraperitoneal, intra-arterial, intrasplenic, mucosal, intramuscular, subcutaneous, transdermal, intradermal, subretinal, intratracheal, intracranial, and others. The main advantage of liposomal gene delivery is the lack of immunogenicity. Therefore, the nucleic acid–liposome complexes can be readministered to the patient without any harm and without compromising the efficacy.

Peptide-mediated gene delivery

Peptide gene carriers have been incorporated with redox-sensitive thiols. Peptides containing cysteine and lysine residues have been developed, for example, Cys-Trp-Lys, which condense the plasmid DNA. The thiol group gets instamtly oxidised which results in the formation of a highly stable complex in vitro.

Polymer-mediated gene delivery

Biodegradable polymers are highly biocompatible with low toxicity. Recently, a water-soluble biodegradable polymer, poly[a-(4-aminobutyl)-L-glycolic acid, has been developed which condenses DNA and leads to DNA release after hydrolysis. This polymer complex has shown high in vitro gene transfection with low cytotoxicity. Recently, other types of biodegradable have also been developed by synthesizing cationic copolymers derived from polyethylene glycol and cationic polyphosphoester, respectively. Thermosensitive polymers have also been developed which leads to controlled release of encapsulated DNA in response to temperature changes.

Applications in dentistry

Gene therapy aims to treat both genetic and infectious diseases by the introduction of new genetic material into the appropriate cells in the body. In the simplest case of a defective gene causing disease, addition of the new gene will restore function. Alternatively the new genetic material can be designed to selectively kill a tumour cell, to induce an immune response, or to protectively ‘‘immunize’’ a cell against an incoming pathogen. Nontherapeutic uses of gene therapy include gene marking, which has proved especially useful in identifying the sources of recurring malignancies in autologous bone marrow transplant patients.

Bone repair

The three basic conditions required for regeneration of bone are enhanced by gene therapy: osteoinduction via expression of growth factors, osteoblast differentiation facilitating laying down of osteoid matrix and utilizing an osteoconductive

apparatus. The property of localised gene delivery to a particular site is suitable for bone formation. Numerous osteogenic factors like IGF-1 and IGF-2, BMPs, PDGF, FGFs and VEGF modulates bone formation through various processes like chemotaxis, proliferation, and differentiation. The most widely studied osteogenic factor showing promising results is BMP-2. A unique feature of gene therapy in bone regeneration is the use of osteoconductive scaffold in conjunction with gene delivery. An ideal tissue engineering scaffold exhibits at least four critical properties: biocompatible, three-dimensional architecture, osteoconductive, and biodegradable. The currently used scaffolds are usually made of natural polymer (collagen, or hyaluronic acid), synthetic polymers (polylactic acid, or polyglycolic acid), and composites (polylacticcoglycolic acid and polypropylene fumarate). (Jeffrey Luo *et al*., 2005)

Salivary gland

The secretions of salivary glands guards and aids in functionality of all oral and upper gastrointestinal tract tissues. Salivary glands are easy to access in a relatively non-invasive manner; are well-encapsulated and the concentration of vector delivered to the gland is not diluted by other body fluids; physiological processes are easy to assess; and are not necessary for life, if a severe untoward complication develops. Gene transfer encoding secretory proteins into the salivary glands can be potentially used to treat both local (upper GI) and systemic disorders as salivary glands can secrete proteins in endocrine as well as exocrine fashion. The key to potential application of this technique lies in the ability to produce therapeutic levels of the transgenic protein and its secretion in the correct direction. Saliva's natural defence mechanisms are augmented by the secretion of protein in an exocrine direction. Also, single protein deficiency disorders can be corrected by utilising the capability of salivary epithelium to produce transgenic proteins and its subsequent secretion into the bloodstream. (Skálová *et al*., 2010; Baum *et al*., 2002)

Oral Cancer

Oral cancer is a genetic disease in which the genes that control cell growth and apoptosis are mutated, allowing cells to acquire the ability to invade and metastasize. Oral Cancer is a good candidate for gene therapy because primary and recurrent lesions are readily accessible for injection or application of the agent. The various approaches include – immunomodulation through cytokines, immune accessory molecules or tumour antigens using immunogenic therapy; introduction of genes with antiangiogenic properties using antiangiogenic therapy; selectively killing tumour cells using oncolytic virus therapy; introduction of tumour suppressor genes by gene replacement therapy; and suicide gene therapy for cancer cells through converting a prodrug into an active form that is toxic for target cells. (Xi and Grandis, 2003)

Pain

Studies on mice have found that transfer of genes expressing an endogenous opiod into the spinal fluid can produce similar effects like an opiate analgesic. Dorsal root ganglia was the target site as it acts as a "pain gate" by interrupting pain signals from the body towards brain. The virus blocks the pain signals by using the host cells' machinery to dissolve the opioid protein. (Rice, 2008)

Gene transfer to Keratinocytes

Oral keratinocytes have certain features that facilitates the gene therapy techniques

1. Presence of stem cells maintains the gene transfer over a prolonged duration.
2. Site specificity can be achieved by using keratinocyte-specific promoters.
3. Good access for direct gene delivery and grafting for in vitro therapy.
4. Secretory capacity of keratinocytes can be harvested for local or systemic distribution of gene products. (Baum, 1995)

Orthodontic tooth movement

Orthodontic tooth movement by activation of osteoclasts through the transfer of RANKL gene to the periodontal tissue has been described. Local gene transfer maintains the appropriate concentration effectively and prolongs the protein expression, along with avoiding the systemic effects. Research has been done to inhibit the orthodontic tooth movement too, which can be used as an important tool for anchorage reinforcement and stability. Local osteoprotegerin gene, produced by osteoblasts enhancesrepair of external root resorption during retention. However, the exact biological mechanism behind this is still not clear and more studies are needed to elucidate the role of RANK / RANKL / OPG axis in repair. (Kanzaki *et al*., 2006)

DNA vaccination

Gene delivery has emerged as a potential and novel way of vaccination. Specific salivary IgA and IgG have been secrteted in the salivary gland tissue of mice through immunization of gland using plasmid DNA encoding the Porphyromonasgingivalisfimbrial gene which prodecesfimbrial proteins. Secretory IgA produced in saliva could hypothetically limit the ability of P.Gingivalis in plaque formation by neutralising it. Additionally, fimbrial proteins in saliva inhibit the attachment of P. gingivalisto the plaque by binding to the pellicle. Although, application of gene delivery for vaccination are in the native stage, it is still potentially reasonable to propose that this is a futuristic strategy for preventing periodontal diseases and dental caries. (Schalk *et al*., 2006)

Conclusion

There is now a considerable body of published literature to demonstrate the feasibility of using gene delivery for clinical applications.

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