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Systemic Delivery of Genetic Material by Non-Viral Vectors

P. TYAGI, J. ALI*, ALKA AHUJA, SANJULA BABOOTA AND R. K. KHAR
Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi-110062.

This article introduces the reader to a number of ventures incorporated in the field of non-viral gene delivery and also the applications of these vectors in the field of gene therapy. The near completion of human genome project has led scientists to search for novel techniques to deliver the newly found genes, which in turn are responsible for a disease. Vehicles for gene delivery *in vivo* can be divided into viral and non-viral vectors. Non-viral gene delivery involving the use of cationic lipids and polymers still continues to enjoy a high profile due to the safety and advantages offered by these systems when compared to viruses. The clinical use of non-viral gene delivery systems in cystic fibrosis or cancer has involved their direct application to the site of pathology due to targeting difficulties experienced.

The Human Genome Project has substantially increased our knowledge of the molecular mechanisms of various diseases[1]. In contrast to conventional therapies, the human genome project has envisaged a modern era with potential for profound impact on the treatment of incurable diseases such as cancer, cystic fibrosis, neurological disorders and AIDS. Need of novel treatment methods has risen out of the general limitations seen in conventional methods. Poor specificity of chemotherapeutic agents leads to the suppression of the bone marrow and other fast dividing cells thus limiting its use. Another drawback of the chemotherapy is seen in the development of resistant phenotypes, which in turn make the therapy inefficient.

Delivery of genetic material (DNA or RNA) represents a much more efficient and specific therapy, due to its ability to directly influence the defective genes. A deficient gene can be cured either by replacement of the defective or by introducing a gene that counteracts the deficient gene. Unfortunately, despite the plethora of research going on in various gene delivery laboratories, commercial gene therapy is a far-fetched dream. Numerous difficulties are still to be overcome before efficient clinical application is attempted. An elusive search is on for an efficient method to transfer a therapeutic gene into the target cell *in vivo*.

Till date, two different approaches have been utilized for the delivery of genetic material. They can be broadly classified as viral and non-viral delivery systems. Viruses were the first to be exploited to deliver genetic material, as the entry of foreign nucleic acid into a host cell is a regular event in the life cycle of a virus. The first viral vector to be adapted as a gene therapy vector was the retrovirus, as they can efficiently adsorb to and enter a target cell. The drawback associated with the use of retrovirus is that it can only be targeted to cells that are undergoing cell division. Other viral vectors exploited include lentivirus, adenovirus and adeno-associated virus and herpes virus. Each virus has its own share of disadvantages, which outweigh its advantages and ultimately make their use unfeasible (Table 1). Viral vectors have limitations because they provoke mutagenesis and carcinogenesis. Repeated administration of viruses induce an immune response resulting in uptake of plasmid DNA by mononuclear phagocytic system, which in turn abolishes the gene expression as in case of anticancer gene therapy where the antisense and ribozyme therapy tend to down regulate the expression of a particular gene by interacting with mRNA.

*For correspondence
E-mail: javedaali@yahoo.com

Considering the disabilities of the viral vectors, a number of approaches have been developed which offer promise for the efficient introduction of DNA into cells without the problems associated with viral components.

NON-VIRAL VECTORS

Various types of non-viral vectors have been proposed so far, and they can be broadly classified into two broad categories, naked DNA delivery by a physical method and delivery by a complex of DNA with a carrier[2]. In the former case, DNA can be directly delivered to the cytoplasm, while DNA cationic carrier complex requires an endosomal and/ or lysosomal release (since it is entrapped in these organelles after its cellular uptake).

Naked DNA:

As early as in 1990, it was found out that the application of plasmid DNA resulted in gene expression in the skeletal muscle[3]. Besides the skeletal muscle, direct DNA application has resulted in expression in liver[4], thyroid[5], heart muscle[6], brain[7] and epidermis[8]. This quality of muscle transfection has led the naked DNA injection to be used for vaccination purposes, where DNA encoding for an antigen is administered to stimulate an immune response.

Uptake of the naked DNA can be increased by a simple mechanical massage. The procedure was performed by placing both thumbs over the upper abdomen of a mouse directly underneath the rib cage and pushing the liver against the back of the animal[9]. It is expected that gene transfer by massage follow a pressure mediated uptake. A large pressure gradient being built across the cell membrane might facilitate DNA uptake[10]. Lately, occlusion of capillaries has been found to be equally useful to increase free naked DNA uptake. The basis of this model is that retention time is increased if the blood flow to the target organ could be restrained. Occlusion has increased gene expression for liver gene delivery by putting a simple clip either at junction of hepatic vein and caudal vena cava or a clip at the junction of portal vein and hepatic artery.

DNA cationic liposome complexes:

Liposomes are microparticulate or colloidal carriers usually 0.05-5.0 μm in diameter which form spontaneously when certain lipids are hydrated[11]. Cationic liposomes are made up of cationic lipids such as dimethyl dioctadecyl ammonium bromide (DDAB) and 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP). Such lipids are of importance as they can be used to deliver negatively charged molecules (DNA, RNA and oligonucleotides). The basis of this approach is that positive charge of the lipids interacts with the negatively charged cell membrane and perhaps gain entry by receptor mediated endocytosis. However as cell toxicity has been reported with cationic liposomes, research is still required to reduce the toxicity of these carriers. Stability is also an important factor for liposomes and lyophilisation techniques are being employed to improve the biological properties of lipid carriers[12].

Polymers:

Polymers that can be protonated at a physiological pH have been employed as gene delivery carriers. The electrostatic attraction between the cationic charge on the polymer and the negatively charged DNA results in a complex. Polymers such as poly L-lysine[13], polyethyleneimine[14], poly L-histidine and chitosan[15] have been used for gene delivery. Polymers display striking advantages as vectors as they can be specifically tailored for multifarious applications by choosing appropriate molecular weights and coupling of targeting moieties. Production of polymers at large scale is also rather easy as well.

Poly L-lysine was one of the earliest polymers to be used for the purpose of gene delivery as it has the advantage of being biodegradable. Polyethyleneimine has the advantage of providing protection against nuclease degrada-

TABLE 1: GENE THERAPY BY VIRAL VECTORS

Vector	Advantages	Drawbacks
Retrovirus	Long term expression and high efficiency to transfect	Requires dividing cells and limited size of material can be loaded,
Adenovirus	Broad range of target cells and no cell division required	Short term expression and Immunogenic, Cytopathic effects of virus
Adeno-associated virus	Site specific integration and no dividing cells required	Limited size of DNA insert

tion, possibly due to their higher charge density and more efficient complexation[16]. Chitosans are biodegradable linear aminopolysaccharides derived from chitin. Depending on their molecular weights, chitosans are capable of forming small (<100 nm) complexes with plasmid DNA. Other polymers used are dendrimers and Pluronic® polymers. Dendrimers are spherical, highly branched polyamidoamines whereas Pluronic® copolymers are a combination of ethylene oxide and propylene oxide. Besides these major techniques discussed above various other methods have been developed and are under investigation for the use of gene delivery. Other methods of gene delivery are as follows.

Electroporation:

In 1988, it was found that electric pulses can cause reversible permeability changes in cell membranes and consequently can be used to entrap extracellular molecules[17]. This technique has been used for transfecting DNA into tissues such as skin[18], liver[19] and melanoma[20]. *In vivo* gene delivery has been achieved using electroporation so far. The electric pulse opens up pores in the cell membrane through which DNA can pass down a concentration gradient into the interior of the cell. It has been seen that parameters such as electrical field strengths and time of duration greatly affects the transfection efficiency. Skeletal muscle requires mild (100 to 400 V/cm) electrical field strength for improved transfection.

Lipid-polymer-DNA complexes:

These have been prepared by condensing DNA with a polycation such as poly L lysine and then entrapping this polymer DNA complex into an anionic or neutral liposome. Such lipid-polymer DNA complexes results in better gene transfer and lower toxicity when compared with cationic liposomes alone.

Ultrasound-mediated delivery:

Ultrasound-facilitated delivery is one of the approaches used to improve the efficacy of *in vivo* gene therapy using mechanical force. A significant increase in transgene expression was observed when insonation of 20 W/cm^2, 1 MHz was given after injection of plasmid DNA[21]. As ultrasound has been generally regarded as safe and harmless to the human body, this technique offers a relatively safer method and can be widely applied clinically. This approach is also useful as a site-specific delivery method, which can be, attained by insonating a specific area only.

Light-induced gene delivery:

Light-induced technology, also known as photochemical transfection, makes use of photosensitizing compounds that localize mainly in the endosomes and lysosomes. When light is induced, these membrane structures will be destroyed and thus release the endocytosed DNA into the cell cytosol. Several photosensitizers have been used (such as aluminium phthalocyanine) and light activation of such sensitizers may result in cytosolic release of lysosome-entrapped macromolecules[22].

Nanoparticles:

The use of L particles for the efficient and specific transfer of a gene or drug into human hepatocytes both in culture and in a mouse xenograft model was studied. In this model, intravenous injection of L particles carrying the gene for

TABLE 2: SUMMARY OF VARIOUS TARGET MOIETIES AND THE CELL TYPE AFFECTED BY NON-VIRAL VECTORS

Target under investigation (Disease/tumor)	Gene being delivered	Lipids being used
Cystic fibrosis	CFTR gene	DOTAP
Melanoma	MHC gene	DMRIE/ DOPE
Breast and neck cancer	E1A gene	tgDCC
Ovarian and breast cancer	E1A gene	DC-Chol
Cutaneous metastases of human carcinoma	MHC gene	DC- Chol/DOPE
Hepathic metastases of colorectal carcinoma	HLA-B7	DMRIE/DOPE

DOTAP-1,2-dioleoyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide; DMRIE- 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide; DOPE-Dioleoyl phosphatidylethanolamine; DC-chol-3beta(N-(N',N'-dimethylaminoethane)-carbamoyl) cholesterol; tgDCC-3beta(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol/dioleoyl phosphatidyl- ethanolamine; CFTR- cystic fibrosis transmembrane conductance regulator; MHC- Major histocompatibility complex.

green fluorescent protein (GFP) or a fluorescent dye resulted in observable fluorescence only in human hepatocellular carcinomas but not in other human carcinomas or in mouse tissues. When the gene encoding human clotting factor IX was transferred into the xenograft model using L particles, factor IX was produced at levels relevant to the treatment of hemophilia B[23].

Different lyoprotective agents (LPA) for the minimization of particle aggregation were studied. Core shell silica particles with a hydrodynamic diameter of 28nm, an IEP of 7.1 and a zeta potential of +35mV at pH 4.0 were synthesized. The activity of the nanoparticles was measured as DNA-binding capacity and transfection efficiency in Cos-1 cells before and after lyophilisation. It was found that massive aggregation occurred in the absence of LPA. Of the various LPAs screened in the present investigations, trehalose and glycerol were found to be well suited for conservation of cationically modified silica nanoparticles with simultaneous preservation of their DNA-binding and transfection activity in Cos-1 cells[24].

APPLICATIONS OF NON-VIRAL GENE THERAPY

Anti cancer gene therapy:

Cancer has been the most sought after disease in terms of treatment by gene delivery as site specificity is seriously expected of cancer chemotherapy. Cancer gene therapy can be utilized in various ways i.e. by supplying a copy of mutated tumour suppression gene, by supplying an anticancer prodrug-activating gene, or by supplying an enzyme encoding for an antigen[25]. A gene encoding for an antisense RNA can be delivered to inactivate a mRNA. Inactivated mRNA can thus not be translated successfully and the expression of the targeted gene is hindered. Genes encoding for ribozymes can also be delivered. Tumour suppression has been noted in many immunodeficient mice when treated with liposomal formulations[26]. Clinical trials have also been carried out using liposomal and naked DNA gene delivery strategies. Naked DNA has been used in hepatocellular carcinoma patients using p53 gene in the form of naked DNA. Liposomes have been used in melanoma patients using genes encoding for HLA-B7.

Immunization:

The predominant and most frequently described application of gene delivery is in DNA immunization. In DNA immunization, genes encoding for a tumour antigen is introduced in the body, in order to elicit a cellular or humoral immune response e.g., plasmid DNA encoding Leishmania antigens is able to protect susceptible mice from Leishmaniasis. Various routes of administration have been investigated for DNA immunization (or vaccination). Oral administration of chitosan-DNA nanoparticles resulted in a protective immune response in a murine peanut allergy model[27]. Additionally widespread mucosal immunity has been observed in animals administered intranasal naked DNA.

Cystic fibrosis:

Cystic fibrosis is one of the main single gene deficiency diseases[28]. A gene, by the name of cystic fibrosis transmembrane regulator (CFTR), is found to be mutated in the patients of cystic fibrosis. CFTR gene is known to be the controller of chloride membrane transport. This causes an accumulation of thick, sticky mucus in the respiratory tract and digestive tract, which leads to recurrent pulmonary lung infections and pulmonary damage[28]. A full restoration of defective chloride transporter was reported in some animals when cystic fibrosis mouse model were administered the CFTR gene in a liposomal formulation[29]. In human clinical trials some restoration of a functioning chloride channel has been reported[30].

Prenatal gene therapy:

It is the curing of disease during pregnancy to prevent the postnatal manifestations of genetic disease in the newborn. These studies could lead to therapies for the correction of genetic defects in embryos and fetuses. In a study on pregnant mice the gene expression was also seen in the progeny, though at a low level. Cationic liposomes were used for this study[31]. Prenatal gene therapy is still a distant dream, as ethical and social questions will prevent research in this area.

Gene therapy for rheumatoid arthritis:

Rheumatoid arthiritis is an inflammatory disease characterized by synovial inflammation and erosion of articular cartilage and subchondrial bone[32]. Thus an efficient vector for gene therapy of rheumatoid arthiritis would be one that is capable of efficiently delivering the gene of interest to the target cell in the diseased synovium. Interleukin 10 gene when injected into an arthiritic mouse model resulted in the decrease of the inflammation[33]. However, a major drawback in gene therapy of rheumatoid arthritis is the low uptake by synovial fibroblasts and in synovial cells after intra-articular injection[34]. Some success has been achieved in the animal models of arthritis using naked plasmid DNA[35,36].

Gene therapy for cardiovascular disease:

Gene delivery to the myocardium has been achieved by using direct DNA injection method. Using this method, transfection of gene in the myocardial cells has been achieved. Gene transfer of two different plasmids by direct intracardiac injection has been achieved[35]. In addition, patients have been administered vascular endothelial growth factor complexed with cationic lipids in an effort to prevent myocardial ischaemia[38].

Gene therapy for AIDS and other infectious diseases:

Negative stranded oligonucleotides can be used to bind, by standard Watson-Crick base pairing, to target genes (usually RNA). Antisense oligonucleotide is the name given to such binding as it results in physical interference with target transcription or translation sites. Besides antisense oligonucleotides ribozymes have also been used for infective organisms. Ribozymes are enzymatic moieties that cleave the RNA target once a duplex RNA-RNA has been formed by antisense oligonucleotides. Ribozymes have been effective at reducing the protein expression of HIV-1 genes[39-41]. Suicide genes have also been used to target infectious organisms. Suicide genes are designed to produce the death of the infected cell.

Gene therapy for neurological diseases:

Gene therapy approaches for neurological disease may be used to protect against neurological disease, to slow the progression of a disease, or to assist in repair of damaged tissue. Diseases such as multiple sclerosis, Guillain-Barre Syndrome, Parkinson's disease, Huntington's disease and Alzheimer's disease have been investigated as potential candidates for gene therapy. In Parkinson's disease, a decrease in dopamine results in a disorder affecting brain controlling voluntary movement leading to tremor and muscle rigidity. Tyrosine hydroxylase (TH) encoding liposomes have been used for gene therapy of Parkinson's disease[42,43].

Currently, most of the candidate diseases are incurable and some such as cancer are widespread. Non-viral delivery systems such as liposomes and polymers have been attempted but unsolved problems also remain with these systems. Improvements in all aspects of these delivery systems are required if efficient systems are to emerge. Results of various animal model studies demonstrate that real clinical benefits can be attained by these non-viral systems. Since interest in gene therapy has dramatically increased in the past few years it is likely that all the techniques being developed will eventually merge with all the other aspects of medical treatment. A multidisciplinary consortia consisting of chemists, pharmacologists, molecular biologists and others will be the way ahead.

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