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Gene Medicine : A New Field of Molecular Medicine

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Gene therapy has emerged as a new concept of therapeutic strategies to treat diseases which do not respond to the conventional therapies. The principle of gene therapy is to introduce genetic materials into patient cells to produce therapeutic proteins in these cells. Gene therapy is now at the stage where a number of clinical trials have been carried out to patients with gene-deficiency disease or cancer. Genetic materials for gene therapy are generally composed of gene expression system and gene delivery system. For the clinical application of gene therapy in a way which conventional drugs are used, researches have been focused on the design of gene delivery system which can offer high transfection efficiency with minimal toxicity. Currently, viral delivery systems generally provide higher transfection efficiency compared with non-viral delivery systems while non-viral delivery systems are less toxic, less immunogenic and manufacturable in large scale compared with viral systems. Recently, novel strategies towards the design of new non-viral delivery system, combination of viral and non-viral delivery systems and targeted delivery system have been extensively studied. The continued effort in this area will lead us to develop gene medicine as "gene as a drug" in the near future.

Key words: Gene therapy, Delivery system, Viral, Non-viral

INTRODUCTION

Gene therapy has emerged as a new field of therapeutics for the treatment of variety of genetic and acquired diseases at molecular level. Since its first application in clinical trials in 1989, gene therapy has become a promising approach for the treatment of a number of diseases, which do not respond to the conventional therapy (Tomlinson and Rolland, 1996).

The goal of gene therapy is to treat diseases by introducing genetic materials into patient cells to induce the production of therapeutic proteins from these cells. Therefore, the key features that allow gene therapy include the effective and prolonged gene expression after administration of genetic materials, to achieve therapeutic level of protein. These features depend on the elaborate design of genetic materials, which are generally composed of both a gene expression system that contains a therapeutic gene and a synthetic gene delivery system.

It is critically required to develop genetic materials as a "gene medicine" to apply in clinics in a way which conventional pharmaceutical products are used (Ledley, 1995; Mahato *et al.*, 1999). Drastic advances in molecular biology, biochemistry, genetics and pharmaceutics have accelerated the steps to meet the needs for developing gene medicine. This article will review the principles underlying gene therapy, the status of current gene therapy technique, clinical applications of gene therapy and the challenges for developing improved gene medicine in the future.

PRINCIPLES OF GENE THERAPY

Gene therapy employs the approach of 'gene addition' wherein a malfunctioning gene activity can be augmented by the induction of genetic materials containing a correct copy of the gene into the non-functioning somatic cell (Romano *et al.*, 1999; Wadman, 1998). An ideal gene therapy procedure using genetic materials would fulfil the requirement wherein;

i. genetic defect is being corrected but not replaced.
ii. the delivery system do not elicit any immunogenic response.
iii. the transfection of DNA is limited to the dysfunctional cells.

For the effective gene transfer, after administration of

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genetic materials, the genes should reach the target cells without degradation in the serum or at the injection site. The genes should then contact with the target cell, interact with the cellular membranes, and subsequently undergo internalization or cytoplasmic delivery inside the cells. As a last step, the genes must cross the nuclear membrane and be processed for efficient transcription and translation. However, when genetic materials are administered *in vivo*, they inevitably encounter the biological barriers inside the body and inside of the cells.

Barriers to gene delivery

Barriers in the body

The barriers that genes encounter after administration are dependent on the route of administration (Kabanov, 1999). In case of systemic administration, for example, particulate non-viral delivery systems are easily eliminated by reticuloendothelial system. Interactions with components present in the body fluids, such as serum proteins, can strongly affect the *in vivo* fate of genes delivered with non-viral vector, thus modifying its tissue distribution. In certain cases, genes cannot reach the target organ due to the natural biological barriers such as blood-brain barrier (Chung and Choiocca, 1998) or intestinal barrier. Degradation of vector itself by enzymatic digestion by nuclease can also be the barrier to impede gene transfer. From this point of view, it is important to develop gene delivery systems, which can remain stable until they reach the target site and then be able to release DNA during the interaction with target cells.

Barriers in the cells

The intracellular environment, where genes meet after reaching the target cells, are getting increasing attention since the transgene may be sequestered within the wrong compartment of the cell, thus leading to degradation instead of trafficking to its intracellular target (Tseng *et al.*, 1997).

Genes delivered by non-viral vector generally enter cells through endocytic process, and then they are subjected to subsequent intracellular vesicular trafficking. Transgene digestion by lysomal compartments should be overcomed for the effective gene transfer.

Components of gene medicine

The essential components of gene medicine are as follows:

i. Therapeutic gene that codes for a protein. Genes can be bacterial, viral, animal, or plant in origin.
ii. Regulatory component to control the expression of therapeutic gene.
iii. Gene delivery system

For the successful gene therapy, the efforts to design the effective gene expression regulation system and gene delivery system (Russel, 1997) have been extensively made and will be discussed in this review.

DISEASES FOR GENE THERAPY

Gene therapy was applied for the treatment of genetic, especially monogenic, disorders at the first time. In the recent years, as a result of dramatic advancement and closer cooperation among various disciplines such as molecular biology, biochemistry, genetics, etc., the scope of gene therapy has been expanded and now it is no more restricted to the genetic disorders (Anderson, 1998; Karin, 2000). Rather it is now getting extended to the treatment of acquired diseases such as cancer, AIDS, Alzheimer's disease, cardiovascular diseases, neuronal injury, rheumatoid arthritis and other infectious diseases. Table I summarizes the candidate diseases in gene therapy.

CLINICAL TRIALS

The inception of this new concept of therapy has been advanced to the stage of clinical trials in humans. The first clinical trial in the gene therapy was for the treatment of adenosine deaminase (ADA) deficiency (Kantoff *et al.*, 1986). At present, there are more than 300 clinical trials, involving about 3500 patients, being conducted throughout the world (Human gene therapy protocols, 2000). The results of these clinical studies, in particular the ones attempting to correct the defective genes, have not been satisfactory yet; only a handful of patients have benefited from this technology. Therefore, the efficacy of gene therapy has not been clinically proven yet. The safety and ethical issues about manipulating human genes are still of general concern and are gaining more significance after the publication of the reports on the loss of human lives during clinical trials with gene therapy (Verma and Somia, 1997; Walsh 2000). Even it was claimed that the clinical trials with gene therapy should be stopped unless the risks to the patients or general public have been duly addressed and alleviated (Anderson, 2000; Walsh, 2000). On the other hand, there are evidences proving that the concern about that gene therapy may modify human germline cells or the zygote is unnecessary (Russell, 1997; Wadman, 1998).

With all of this murky perspective, there is no doubt about the strong potential of gene therapy as a novel therapeutic strategy for treating diseases, which do not respond to the conventional therapy. This situation demands more careful, systematic and scientific approach in gene therapy. It underscores the need for improvement in many research areas, including vector designing, gene expression and understanding the disease mechanism to ensure the accurate diagnosis of diseases.

APPROACHES FOR GENE THERAPY

Several different approaches have been performed for gene therapy, depending on the gene-administration methods.

Table 1. Diseases for applying gene therapy strategies

Disease	Defect	Target cells (strategy)	References
Genetic			
Severe combined immunodeficiency	Adenosine deaminase 4 (ADA)	Bone marrow cells or T-lymphocytes	Cavazzana-Calvo *et al.*, 2000 Buckley, 2000
Heamophilia	Factor VIII (A type) or Factor IX (B type) deficiency	Liver, muscle, fibroblasts or bone marrow cells	Habeck, 2000 Thompson, 2000
Familial hypercholesterolaemia	Deficiency of LDL receptor	liver	Ponder, 1999
Cystic fibrosis	Faulty transport of salt in lung epithelium, Loss of CFTR gene	Airspaces in the lung	Simakajornboon and Davis, 1998 Ferrari *et al.*, 1999
Haemoglobulinpathies:thalassaemias/sickle-cell anaemia	α or β globulin gene	Bone-marrow cells, macrophages	
α1-antitrypsin deficiency: Inherited enphysema	α1-antitrypsin	Lung or liver cells	Geddes and Beckles, 1995
Acquired			
Cancer	Many causes	Many cell types Ex) *DNA vaccination* Immunomodulation -Cytokine genes (TNF, IL, IFN-γ, GM-CSF) Dendritic cells/Prostate cancer	Nanni *et al.*, 1999 Altenschmidt *et al.*, 2000 Albini *et al.*, 2000 Guy *et al.*, 2000 Chang *et al.*, 2000 Gong *et al.*, 1998
Neurological diseases	Parkinson's, Alzheimers, Spinal-cord injury	Direct injection into the brain, neurons, glial cells, Schwann cells	Zou *et al.*, 1999 Latchman, 2000
Cardiovascular	Restinosis, arteriosclerosis	Vascular endothelial cells, Arteries	Alexander *et al.*, 1999
Infectious diseases	AIDS, hepatitis B, tuberculosis	T cells, macrophages, liver	Zoulim and Trepo, 1999 Morris *et al.*, 2000
Liver cirrhosis	Fibrogenesis	Muscle cells/hepatocyte growth factor	Fujimo and Kaneda, 1999
Autoimmune disease	Lupus, diabetes	MHC, β2-microglobulin	Varley *et al.*, 1998

In vitro approach

The in vitro transfection methods for cultured cells use appropriate reporter genes such as CAT, β-galactosidases, luciferase, green fluorescence protein and alkaline phosphatase, complexed with delivery systems, to evaluate the transfection efficiency of target genes. These methods have been used most frequently and have been an important model for the research to improve the transfection efficiency by the cells. However, the in vitro results are not always sufficient for the prediction of *in vivo* behavior after administration of gene medicine to patients.

Ex-vivo approach

This technique is also known as the cell-based approach. In this technique, the gene medicine is given to cells, which are already taken outside from the body of patient. The gene-engineered cells are transferred back to the patient (Ledley, 1996). It is based on tissue transplantation technique and can be applied to any type of tissues or cells which are transplantable (Nakanishi, 1995). The biological barriers encountered within the body as discussed above can be avoided by this approach.

Ex-vivo method has been extensively and successfully employed to transfect keratinocytes, endothelial cells, fibroblasts, bone-marrow cells, peripheral blood cells and hepatocytes (Martin and Murray, 2000 and Puls and Minchin, 1999), because these cells can be easily removed and returned. This technique can also be used for cancer cell treatment and cancer vaccine development (Simons and Mikha, 1998; Leitner and Ying, 2000).

This approach is more patient-specific in majority of the cases since it involves the autologus transplantation of cells. However, allogenic tissue cells may be transplanted as well by applying genes in appropriate delivery systems such as microcapsule (Hughes *et al.*, 1994). The transfection result can also be variable by the interference of types and confluency of cells and transfection procedure. The cost and the labor for the surgery and cell culture remain as an another disadvantage of the *ex vivo* approach.

In situ approach

Genes are given to the target tissues through direct injection or perfusion with catheters in this technique. Examples are the infusion of adenoviral vectors into the trachea and bronchi of patients with cystic fibrosis, the injection of a vector carrying the gene for a cytokine or a toxin, or the injection of a vector carrying the gene directly into the muscle of a patient with muscular dystrophy. This approach is more practical compared with ex *vivo* approach.

In vivo Approach

In vivo approach, genes are injected directly into the blood stream of patients. *In vivo* transfection can be applied to a large variety of types of cells and tissues (Herweiger *et al*., 1995). However, the possibility that the genes are also introduced into reproductive cells can not be excluded and may cause some ethical problems (Wadman, 1998). The transfection efficiency by *in vivo* approach is influenced by many parameters inside the body where numerous biological barriers hinder the access of the delivered gene to the cells (Bally *et al*., 1999). Furthermore, the gene expression is generally transient. However, Further improvement of delivery systems will accelerate the development of effective *in vivo* gene transfer technique.

GENE DELIVERY SYSTEM

Genes can be transferred by physical methods such as microinjection, particle bombardment and electroporation (Feltquate *et al*., 1997; Harimoto *et al*., 1998). The transfection of DNA may also be carried out using naked DNA or by attaching it with a gene delivery system, which is also known as vector. The effectiveness of any gene therapy method primarily depends on the characteristics of gene delivery system, which determines the selectivity and effectiveness of gene transfer to the target cell (Nabel, 1999). Therefore, most of the research in gene therapy to date has been focused on the design of effective vector (Li and Hung, 2000; Peng and Vile, 1999; Hart, 2000). The goal of development of gene delivery system is to increase the gene transfer efficiency by controlling the physicochemical, pharmacokinetic and pharmacodynamic characteristics of gene medicine. Ideal gene delivery system should fulfill the requirements summarized in Table II. Currently, there is no one perfect delivery system that can meet all the requirements for an ideal delivery system. However, researches ongoing worldwide are promising the achievement of this goal in the near future.

The currently available gene delivery systems may be classified as;

i. virus based systems
ii. non-virus based systems.
    a. physical mechanisms
    b. chemical mechanisms.

Each of viral and nonviral gene delivery systems delivers genes to the target cell and directs the uptake of the genes into the nucleus by its unique mechanism. The general advantages and disadvantage of viral and nonviral gene delivery systems are summarized in Table III.

Types of Gene Delivery Systems

Naked DNA Injection

Naked DNA injection is the most convenient and cost-

Table II. Requirements for an ideal vector

- Non-toxic
- Non-immunogenic even after repeated administration
- High transfection efficiency
- Injectable
- Regulatable
- Site-specific targeting possible
- Reliable and longer duration of gene expression
- Ease of production on a large scale
- High capacity in DNA insertion size
- Ability to remove/replace defective genes
- Reproducible
- Stable
- Cost-effective

Table III. General advantages and disadvantages of viral vectors for gene delivery.

Gene delivery system	Types	Advantages	Disadvantages
Viral	Adenovirus, adenoassociated virus, retrovirus, Herpes simplex virus, .etc	Higher transfection efficiency	Restriction in the size and structure of gene to be inserted triggers immune response lack of target specificity Problems in safety, toxicity and large scale production Chances of permanent integration in to the host cell genome. Risk of tumorigenesis
Nonviral	Cationic lipid, liposome, emulsion, polymer, endo-somal lysis peptides, etc	Easier manipulation and large scale produciton Flexibility in the DNA insertion size More specific Targeting Safety, non-immunogenecity Non-pathogenecity	Lower transfection efficiency Shorter gene expression Unstability by serum components in vivo

effective method of gene transfection (Wolff *et al.*, 1990). Naked DNA gives virtually no transfection *in vitro*, but efficient uptake has been observed after intramuscular and intravascular injections, although the mechanism is unknown (Liu *et al.*, 1999). Therefore, the interest in this technique has been developed for raising antiviral immune response by intramuscular injection of the plasmid encoding the viral antigens. Naked DNA itself may not provoke any immune response but the presence of CpG motifs in the expression cassette may trigger the release of cytokines in the recipient leading to broader immune system stimulation (Kreigh, 1999). This phenomenon has been exploited for the application of naked DNA injection in DNA vaccination (Leitner *et al.*, 2000).

The major drawback of naked DNA injection comes from the short duration of gene expression in most tissues. Moreover, this strategy is unsuitable for targeted transfection of genes. A better understanding of the underlying mechanism of DNA cellular uptake *in vivo* may help in further improving the uptake efficiency.

Virus-based systems

Viral infection involves the processes for targeting viruses to specific cells in the body and trafficking the viral DNA into the nucleus. The ability of viruses to enter the cells via specific cell surface receptors and transfer their genetic materials to cellular site for transcription and translation, has led to their extensive use as gene delivery systems (Stone *et al.*, 2000; Tanaka *et al.*, 1998). The virus-based systems have emerged as the most efficient mean to transfect genes into cells (Datwyler *et al.*, 1999; Wu and Attai, 2000). A number of reports have been published where enhanced efficiency for *in vivo* gene transfer has been accomplished by the virus-based systems (Mochizuki *et al.*, 1998). However, there is a concern related to the introduction of exogenous DNA into the host genome (Feuerbach *et al.*, 1996). Furthermore, the use of viral vectors may cause immunogenicity, the size of DNA which can be inserted is limited and the large scale production can not be easily performed. To eliminate the unnecessary function of viruses for the purpose of gene therapy, sometimes the viral particles containing the therapeutic genes are genetically manipulated only to maintain the favorable infective ability.

Several types of viruses have been utilized for the gene delivery. The most commonly used virus based systems include retroviruses, adenoviruses and adeno-associated viruses (Carter, 1999).

a) Retroviruses

Retrovirus contains RNA genome complexed with reverse transcriptase, which can converts RNA genome to the DNA form after it enters the host cells. Retroviral vectors are made by replacing viral genes with foregin DNA sequences, which can then be packaged into new recombinant infectious particles. Retroviral vectors have been widely used for both *ex-vivo* as well as *in vivo* gene transfection procedures. These have also been used in human clinical trials to transduce a number types of tumor cells (Grossman *et al.*, 1992) since the first gene transfer experiment in humans using Maloney murine leukemia virus (Anderson *et al.*, 1990).

Retroviral vectors have the ability to carry even large DNA segments (8 kb) and integrate with the host genome, thus, ensuring long term expression of the transgene. However, for integration of transgene with the host genome, they require the cell to be in a cell cycle (Wu *et al.*, 2000) and thus, they cannot transfect genes to nondividing cells including muscles, brain, lung and liver tissues. Moreover, due to their stable integration with the host cell genome, they may result in the over-expression of the transgene thus causing toxicity and potential oncogenicity.

b) Adenoviruses

Adenoviruses are non-enveloped icosahedron viruses with 36 kb double stranded DNA genome. To date, adenoviral vectors have been one of the greatest promising vector since they have provided unique high efficiency in delivering genes to a various organs. From their superior characteristics, they are often considered in clinical trials for gene therapy (Mehrara *et al.*, 1999).

Gene-deleted, replication-defective adenoviruses have the capacity for up to 7.5 kb exogenous gene insertion (Searle and Mautner, 1998). Recent advancement in the field of adenovirus vector brought the development of second generation 'gutless' adenoviruses, a more advanced vector system which has a capacity to carry up to 35 kb of the transgene (Wang and Huang, 2000).

Unlike retroviruses, adenoviruses efficiently transfect the transgene to both the dividing as well quiescent cells with high degree of expression (Searle *et al.*, 1998). Furthermore, the possibility of random insertional mutagesis and the permanent alteration of the host cell genome is rare. Adenoviral vectors are also non-oncogenic in human beings (Lee *et al.*, 1996). The major limitation to their use, however, is their immunogenic nature as a result of which the neutralizing antibodies are generated against them (Chirmule, 1999). The other limitation is that the induced gene expression is generally transient.

c) Adeno-associated viruses (AAV)

This is a defective member of parvo-virus family. It is a single stranded DNA virus and non-pathogenic to the human beings. The applications of AAV as gene transfer vector have drastically increased in the recent past. The vectors designed from AAV are capable of transducing the genome containing therapeutic genes to a variety types of host cell by various mechanisms (Hirata and Russell, 2000). They can provide sustained transgene expression and have been used in various gene therapy trials (Monahan and Samulski, 2000).

The transfection efficiency by AAV is hardly influenced by the stage of cell cycle and thus they can transduce

genes to both dividing as well non-dividing cells. AAV possess the ability for site-specific integration into the host cell genome. AAV-derived vectors are non-toxic to the host cells and do not induce host immune response. However, their major limitations include the restriction in the insertion size of extraneous DNA (4.5 kb) and the difficulties in manufacturing process.

Several other viral vectors are also currently being evaluated for their potential as carriers for genes. One such example is herpes simplex virus which may be modified to accommodate expression cassette as large as 25 kb and has the ability to transfect large variety of cells. Lentiviral (HIV-based) vectors have an important character of being able to integrate genes into non-dividing cells, but safety concerns associated with the use of these agents remain an area of intense research (Miyoshi *et al.*, 1997).

Non-viral physical systems

Several different physical systems are under investigation for gene transfection to overcome the limitations of viral and non-viral chemical systems (Rols *et al.*, 1998). A recent report has been published in which glass needle-mediated microinjection technique has been adopted for the transfer of transgenes into primary human blood stem/progenitor cells (Davis *et al.*, 2000). The physical methods are gaining attention of researchers due to their simplicity in use, safety and non-toxicity. These physical methods make the DNA to pass through the biological barriers and directly enter the cells, thus avoiding the enzymatic degradation.

a) Electroporation

The principle of electroporation is that electrical impulses induce an area of transient membrane breakdown through which DNA enters the cytoplasm. The application of electrical impulses to induce cell fusion and intracellular protein and drug delivery has been in practice for many years (Rols *et al.*, 1998). More recently the use of electroporation for *in vivo* gene transfection is under investigation (Jaroszeski *et al.*, 1999; Rols *et al.*, 1998). The technique has been reported to exert high site specificity and negligible effects on the cell viability reflecting non-cytotoxocity.

b) Bioballistic particle mediated gene delivery

Bioballistic particle-mediated gene delivery systems, often referred to as 'needle free injection', has been developed as an alternative for the 'Naked DNA injection' into muscles for genetic vaccination (Fynan *et al.*, 1993). The delivery of bioballistic particles is accelerated by devices such as Intraject which liquid under high pressure to accelerate the particle delivery (Lin *et al.*, 2000). Gene Gun uses high-pressure helium stream to deliver these particles. The working mechanism of these devices is that they accelerate DNA-coated gold particles of up to 1 μm dimension that penetrate the cell membrane to deliver DNA into the cytoplasm (Lin *et al.*, 2000). Gene gun technique is feasible for both *in vivo* and *in vitro* gene transfection (Williamson and Johnston, 1991) and hence it has been widely used in cancer gene therapy and gene vaccination (Hasan *et al.*, 1999; Mathei *et al.*, 1997). It has been reported that single gene gun injection of 1 ug DNA may result in significant levels of gene expression (Yang and Sun, 1995).

Non-viral chemical systems

The safety concerns associated with the use of viral vectors have made the non-viral vectors as an attractive alternative during the last decade (Felgner, 1997; Luo and Saltzman, 2000; Maurer *et al.*, 1999; Rolland, 1998). However, most of non-viral vectors give low transfection efficiency compared with viral vectors (Li *et al.*, 2000, Table III). Efforts to improve the efficiency of non-viral gene delivery systems are focused on the understanding of biological factors that affect the functioning and the fate of transgene *in vivo* (Bally *et al.*, 1999).

The non-viral chemical vectors are generally complex synthetic chemical materials (Garnett, 1999; De Smedt *et al.*, 2000). Due to the fact that an exceedingly large number of terms have been used in this research area, the committee on nomenclature of synthetic gene delivery systems was established to simplify, harmonize and integrate the system for the description of these terms (Felgner *et al.*, 1997). The recommendations of the committee are shown in Table IV.

Lipoplexes and polyplexes share similarity in that DNA

Table IV. Terminology for synthetic gene delivery system.

Terminology recommended	Meanings	Terminology in Practice
Lipofection	Nucleic acid delivery mediated by cationic lipids.	Cationic lipid mediated transfection, Cationic liposome-mediated trans-fection, Liposome mediated transfection, Cytofection, Amphifection, Lipid-mediated transfection.
Polyfection	Nucleic acid delivery mediated by condensation of DNA using cationic polymers.	Transfection of DNA mediated through condensation of DNA using poly-lysine, PEI, dendrimers, polycationic peptides etc.
Lipoplex	Cationic lipid-nucleic acid complex.	Cytosomes, Amphisomes, Liposomes, Nucleolipid particles, Cationinc lipid-DNA complex, Lipid-DNA complex, etc.
Polyplex	Cationic polymer-DNA complex.	Molecular conjugates of DNA with polylysine, PEI, dendrimers, poly-cationic peptides, etc.

is incorporated into a complex as a result of the formation of ion pairs between cationic groups of lipids or polycation and anionic groups of the DNA but they show their own advantages and disadvantages in gene delivery.

a) Lipid-based systems

One of the most promising non-viral approaches involves the use of cationic lipids, which are capable of forming complex with plasmid DNA. Such lipids vary in their structure (Fig. 1), however, lipids for gene delivery are invariably composed of a hydrophobic lipid anchor group linked to a positively charged head group through a spacer residue (Lee and Huang, 1997). Although many cationic lipid-based gene delivery systems are being investigated, no definite structure-activity relationship has been established so far, especially with regard to *in vivo* behavior of the lipid molecules. The lipid-based gene delivery has been mediated through liposomes, microspheres, nanparticles and emulsion based systems (Esposito *et al.*, 1999; Hara *et al.*, 1997).

i) Liposomes

Liposomes are small vesicles of bipolar phospholipids with an aqueous interior. They are efficient carriers of drugs, peptides and proteins. Therefore, they have been extensively studied for decades for the controlled and site-specific drug targeting (Mastrobatistta *et al.*, 1999; Montaldo *et al.*, 1999; Lee *et al.*, 1999; Kim *et al.*, 2000). In the recent past, researchers have shown tremendous interest to exploit advancements in the liposome technology for gene transfection (Barron *et al.*, 1999; Shangguan *et al.*, 2000).

Cationic liposomes are frequently used for *in vitro* as well as *in vivo* gene transfer due to their ability to interact with the negatively charged DNA and cell membrane (Ross *et al.*, 1998; Vitiello *et al.*, 1998). In this technique, plasmid DNA is mixed with cationic liposomes to form liposome-DNA complex due to electrostatic interaction. The complex is capable of interacting with the target cells due to residual charge of the complex (Eastman *et al.*, 1997). The mechanism by which liposomes and DNA interact is mainly dependent on the charge, lipid structure and composition of liposomes. It has now become clear that there is extensive lipid arrangements during the complex formation between DNA and liposomes (Sternberg *et al.*, 1998; Zuidam and Barenholz, 1999). Two fundamentally different models have been proposed for cationic lipid-DNA complexes, an 'external' model in which DNA is adsorbed on to the surface of the cationic liposomes and an 'internal' model in which DNA is surrounded by or 'coated' by a lipid envelop (Sternberg *et al.*, 1994). Both these models rely on electrostatic interaction between the complexing moieties.

The cellular uptake, intracellular trafficking and cytotoxicity are the special considerations of liposome-mediated gene transfer technology (Sakurai *et al.*, 2000). The DNA transfected via liposomes does not integrate in the chromosome, rather it remains as episome and transiently expresses its information. Hence, Gene administration for transfection needs to be repeated for long term expression. Despite the fact that many cationic lipids in liposomal form show good gene transfection efficiency, one of the problems associated with such a system is the lack of physical stability of liposome/DNA complexes. Furthermore, conventional methods of liposome preparation suffer from lower encapsulation efficiency. Rapid degradation of liposomes also have limited their effective application *in vivo* to the direct injection into tumor or tumor feeding capillaries (Caplen *et al.*, 1995).

In order to improve liposome-mediated gene transfer, liposome constitution and its surface characteristics have been modified to enhance cell-liposome interaction and

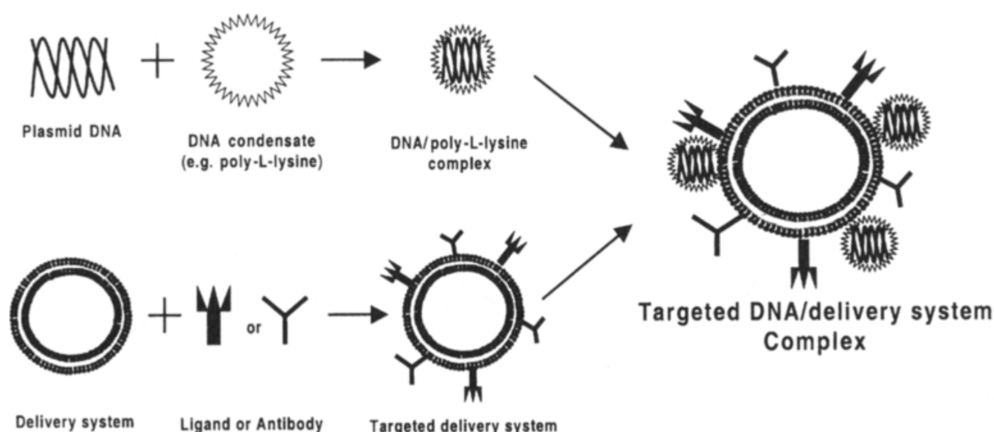

Fig. 1. The structure of representative cationic lipids developed for the application in gene delivery. Abbreviations: DOTMA, N-(2,3-dioleyloxypropyl)-N,N,N-trimethyllammonium chloride; DOTAP, 1,2-dioleyloxypropyl-N,NN-Trimethylammonium chloride; CTAB, cetyltrimethyl-ammonium bromide; DMRIE, 1,2-dimyristyloxypropyl-3-dimethylhydroxy-ammonium bromide; DDAB, dimethyl-dioctadecylammonium bromide; DOSPA, 2,3-dioleyloxy-N-2-(Sperminecarboxamido)ethyl-N,N-dimethyl-1-propanammonium; DOGS, dioctadecylamidoglycylspermine, DC-chol, 3-β-[N-(N,N-dimethylaminoethane) carba-moyl] choleterol.

intracellular trafficking of liposome-DNA complex to the nucleus. Such modifications include the development of pH-sensitive liposomes (Kim *et al.*, 1994), fusogenic liposomes (Noguchi *et al.*, 1998; Watabe *et al.*, 1999), stealth liposomes (Kim *et al.*, 1999; Mok *et al.*, 1999; Ross and Hui, 1999), thermosensitive liposomes (Dass et *al.*, 1997) and immunoliposomes (Bendas *et al.*, 1999).

ii) Emulsions

Recently, researchers paid attention to the application of emulsions as a gene delivery system to overcome the instability of liposomal gene delivery systems after complex formation with DNA or interaction with serum proteins (Felgner *et al.*, 1994; Yi *et al.*, 2000). Emulsions have been widely used as an another colloidal drug delivery systems (Benita and Levy, 1993; Gao *et al.*, 1998; Park *et al.*, 1999). The oil-in water emulsions are made of oil dispersed in an aqueous phase with a suitable surfactant/cosurfactant. Emulsions formulated with soybean oil as a core oil and DOTAP as a surfactant resulted in the formation of stable Emulsion/DNA complexes with significantly enhanced stability in serum (Kim *et al.*, 2000). They also offered another advantages, including the formation of stable emulsion/ DNA complex and the protection from DNAse digestion. The transfection efficiency was also 1.5-3 fold improved compared with cationic liposomes (Liu *et al.*, 1996; Hara *et al.*, 1997). Cationic lipid emulsions formulated with soybean oil and DOTAP more effectively delivered DNA to nasal cavity mucosa compared with commercially available liposomal carriers (Kim *et al.*, 2000).

b) Polymers

Polymers, which have been utilized for gene delivery, include polyethyleneimine, polylysine, polyornithine, polyamidoamine dendrimers, poly(2-demethylamino)ethyl metacrylate and poly (N-alkyl-4-vinylpyridinium) (Esposito *et al.*, 1999; Pouton and Seymour, 1998; Laurent *et al.*, 1999; Cherng *et al.*, 1999; Lim *et al.*, 2000a). Polymers have a potential as a gene delivery system since they provide flexibility in the precise control of physico-chemical properties of gene delivery system. The size, charge and solubility of the complexes formed by DNA and transfecting polycations are strongly dependent on the charge ratio of the polymers and DNA (Kabanov and Kabanov, 1998).

To improve the stability of polyplexes in aqueous dispersion and prevent their interactions with serum proteins, cationic block and graft copolymers have been developed. They include polycations conjugated with nonionic homopolymers, such as poly(ethylene oxide), dextran and poly(N-(2-hydroxypropyl)metacrylamide), for example such as PEO-block-polyspermine, PEO-block-polylysine and PEO-graft-polyethyleneimine (Wolfert *et al.*, 1996; Vinogradov *et al.*, 1998). In some systems, polycations are grafted with amphililic Pluronic block copolymers. This type of polymers exhibit the improved ability to incorporate into cell membranes because of the amphilicity compared with PEO types. Mixing of the conjugate of Pluronic P123-polyethylenemine with free Pluronic P123 has been shown to exhibit high transfection efficiency by forming stable complex with DNA (Kabanov *et al.*, 1998).

Efforts to develop biodegradable polymers led to the synthesis of biodegradable poly-cationic polymers such as Poly(alpha-(4-aminobutyl)-L-glycolic acid) (PAGA) for gene delivery (Maheshwari *et al.*, 2000). PAGA/DNA complexes showed about two fold higher transfection efficiency compared with polylysine/DNA complexes (Lim *et al.*, 2000b). As an another approach to develop more advanced polymeric delivery system, polylysine cationic polymer was modified as (N-Ac-poly(L-histidine)-graft-poly(L-lysine) to provide pH-sensitivity (Benns *et al.*, 2000).

Chimeric delivery system

A new concept of vectors has been established to overcome the limitations of currently available vectors. Several groups are now working on the development of chimeric viral vector systems that combine the favorable attributes of two different viral vectors. These chimeric vectors might allow the researchers to achieve a ideal gene delivery system (Reynold *et al.*, 1999).

For example, gene delivery system in which Sendai virus was combined with liposomes was safe for repeated use due to much less immunogenicity and toxicity compared with the other viral or lipid vectors (Kaneda *et al.*, 1999). To overcome the immunogenicity problem, gutless virus vectors were engineered (Dranoff, 1997). To improve the targeting ability to specific cells, retroviruses were incorporated in liposomes (Nielsen and Maneval, 1998). Many groups have reported the design of chimeric viruses in which AAV is carried by herpes simplex virus (Costantini *et al.*, 1999). Natsume and coworkers (2000) have recently reported that the conjugation of adenoviruses with liposomes reduces the antigenicity of the adenoviral vectors. Use of polymers together with cationic liposomes was also reported to enhance the transfection efficiency. The transfection efficiency of genes trasferred by electorporation was further enhanced by aurintricarboxylic acid (Glasspool-Malone *et al.*, 2000). Coating of cationic nonviral vector with protective, anionic poly(ethyleneglycol) copolymers decreased the undesirable interaction with serum proteins in vivo (Finsinger *et al.*, 2000). Formulation of adenovirus in poly-lactic-glycolic acid microspheres significantly improved the gene transfer while decreasing the immunogenecity frin adenoviruses (Matthews *et al.*, 1999).

Targeted delivery

In the field of gene therapy, the researchers have finally reached to a stage where they are striving for a breakthrough to design a vector which can site specifically target the required gene (Benns and Kim, 2000). The basic aim of gene targeting is to transfect gene to the diseased tissue

or cells without being detrimental to the healthy tissues (Peng, 1999). This strategy gains importance in cancer gene therapy where we are looking for cytotoxic gene products (Cristiano and Roth, 1996; Vile *et al.*, 2000). Furthermore, this will help in reducing the required amount of vector and improve the efficiency of procedure. For establishing targeting strategies, vectors are designed by modification with ligands or antibodies (as described in Fig. 2), which can be specifically recognized by receptors or antigens relatively overexpressed in some cells or some organs (Nielsen and Maneval., 1998; Bendas *et al.*, 1999; Lim *et al.*, 2000a).

Targeted delivery can be applied for both viral as well non-viral vectors (Condreay *et al.*, 1999; Ogris *et al.*, 1998). Ligands including asialoorosomucoid, transferrin, folic acid, epidermal growth factor and fibroblast growth factor (Kim and Jeong, 1997; Nielsen and Maneval, 1998; Kawakami *et al.*, 2000) and antibodies such as antibodies against Her2/NEU and epidermal growth factor receptor has been applied for targeted gene delivery.

Route of Administration

Genes can be administered by systemic, organ or tissue specific or localized delivery. The targetability, transfection efficiency and transfection results are dependent on the route of administration even same type of genetic delivery system is used (Lin *et al.*, 2000; Yukai *et al.*, 2000).

Although naked DNA has little or no activity in the cell culture, it shows exceptionally high transfection activity after intramuscular injection (Wolff *et al.*, 1990). With hydrophilic polymer, intramuscular injection of the complexes of plasmid DNA have given better overall performance although the level of expression after intratumoral injection is generally much lower. Subcutaneous administration of DNA may result in the retention of genetic material at or near the site of injection. This is more evident for larger molecular particulate systems. Intravenous administration exposes the delivery complex to the intricate biological environment and thus various factors reduce the access and binding of the vector-DNA complex in the extravascular site (Hofland *et al.*, 1997). Larger particles (>200 nm) and those bearing net positive charge are rapidly eliminated from the circulation. Positive charge certainly results in excessive plasma protein binding, increased non-specific binding and enhanced phagocytosis.

Regional administration of vector-DNA complex may help to circumvent various problems associated with other routes of administration (Pouton and Seymour, 1998). Intramuscular, intravenous, subcutaneous, intratracheal or intratumoral administration provide a means to target cells within these sites. Regional delivery of formulated vector and plasmid complex may increase transgene expression in these tissues or organs. One typical example of regional administration is the successful expression of CFTR gene in cystic fibrosis after intratracheal administration of DNA-lipid complex. Regional gene delivery to lung has been attractive to many researchers due to the capability of immediate access by inhalation (Curiel *et al.*, 1996).

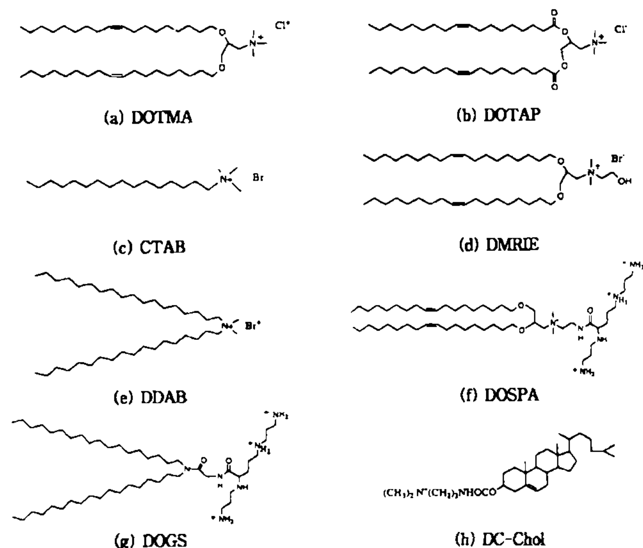

Fig. 2. General structure of a targeted delivery system

REGULATION SYSTEM

Majority of the first generation of vectors were devoid of any regulatory mechanism since the genes which codes for proteins with larger therapeutic indices were chosen for gene therapy (Clackson, 2000). The fact that most diseases are heterogeneous and dynamic whereas most proteins are with low therapeutic indices increased the need for a regulatable vector, which can control the gene expression to maintain the required therapeutic serum concentration of gene expression product (Varley and Munford, 1998). Regulation of gene expression will provide the physicians a means to tailor the gene product to avoid the situations wherein sub or supra levels of the gene expression product may pose threat to the patients safety.

Recent reports have been published where researchers are concentrating on this aspect of gene therapy, referred to as a physiologically responsive gene therapy (PRGT) (Varley *et al.*, 1998). Some typical examples of PRGT under study include glucose-responsive PRGT, hypoxia-related PRGT, drug-related PRGT and cytokine-related PRGT (Mitanchez *et al.*, 1997; Mohammadi *et al.*, 1998). A further step in this direction will be the new technology for light-induced site- directed gene transfection using photosensitizing compounds (Hogset *et al.*, 2000).

EXPRESSION SYSTEM

Plasmid-based gene expression systems, with suitable promoter/enhancer, intron, cDNA and other regulatory

elements, should also be developed in parallel to further enhance the overall gene expression from the complexes between plasmid DNA and delivery system. An important area for the future research will be the development of strong promoter/enhancer system for the transgenes that can induce higher level of transcripted message and translated proteins once the DNA gains access to the nucleus.

CONCLUSION AND FUTURE DEVELOPMENTS

Considerable progress has been made during the last few years in designing more effective gene medicine in terms of transfection efficiency, targetability and safety. However, still the major limiting factor in current gene therapy results from the unsatisfactory efficiency of current gene delivery systems. Continuous efforts should be made to improve the delivery systems to deliver genes more accurately and efficiently to the target sites. It is also imperative to develop vectors that are safe for repeated administrations since in most cases, the transgene expression is transient. Thorough understanding of the mechanism of gene transfer and the factors affecting the transfection efficiency will help the rational design of effective gene delivery systems. Merging of some of the advantages of viral vectors with those of non-viral vectors may be necessary to generate a safe and efficient delivery system. The efforts for the development of regulatory elements that can modulate the promoter activity more accurately and of tissue-specific enhancer-promoter elements should also be pursued, together with the approaches for designing effective delivery systems. A better understanding of gene trascription process is also required.

Taken together, extensive and collaborated research in this area will lead us to develop gene medicine as "gene as a drug" in the near future. It will allow us to extend the scope of diseases which can get clinical benefit from gene therapy.

REFERENCES

Albini, A., Marchisone, C., Del Grosso, F., Benelli, R., Masiello, L., Tacchetti, C., Bono, M., Ferrantini, M., Rozera, C., Truini, M., Belardelli, F., Santi, L. and Noonan, D. M., Inhibition of angiogenesis and vascular tumor growth by interferon-producing cells: A gene therapy approach. *Am. J. Pathol.*, 156(4), 1381-1393 (2000).

Alexander, M. Y., Webster, K. A.., McDonald, P. H. and Prentice, H. M., Gene transfer and models of gene therapy for the myocardium. *Clin Exp. Pharmacol. Physiol.*, 26, 661-668 (1999).

Altenschmidt, U., Moritz, D. and Groner, B., Specific cytotoxic T lymphocytes in gene therapy. *J. Mol. Med.*, 75 (4), 259-266 (2000).

Anderson, W. F., Blease, R.M. and Kulver, K., The ADA human gene therapy clinical protocol. *Human Gene Ther.*, 1, 331-362 (1990).

Anderson, W. F., Gene therapy: the best of times and the worst of times. *Science*, 288 (5466), 627(2000).

Anderson, W. F., Human gene therapy. *Nature.*, 392, 25-30 (1998).

Bally, M. B., Harvie, P., Wong, F. M. P., Kong., Wasan, E. K. and Reimer, D. L., Biological barriers to cellular delivery of lipid based DNA carriers. *Adv. Drug Deliv. Rev.*, 38, 291-315 (1999).

Barron, L. G., Uyechi, L. S. and Szoka, Jr., Cationic lipids are essential for gene delivery mediated by intravenous administration of lipoplexes. *Gene Ther.*, 6, 1179-1183 (1999).

Bendas, G., Krause, A., Bakowsky, U., Vogel, J. and Rothe, U., Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. *Int. J. Pharm.*, 181, 79-93 (1999).

Benita, S. and Levy, M. Y., Submicron emulsions as colloidal drug carriers for intravenous administration: comprehensive physicochemical characterization. *J. Pharm. Sci.*, 82(11) 1069-1079 (1993).

Benns, J. M., Choi, J. S., Mahato, R. I., Park, J. S. and Kim, S. W., pH-sensitive cationic polymer gene delivery vehicle: *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer. *Bioconjug. Chem.*, 18;11(5) 637-645 (2000).

Blau, C. A., Current status of stem cell therapy and prospects for gene therapy for the disorders of globin synthesis. *Baillieres Clin Haematol.* 11(1) 257-275 (1998).

Buckley, R. H., Gene therapy for human SCID: dreams become reality. *Nat. Med.*, 6(6) 623-624 (2000).

Caplen, N. J., Alton, E. W. F. W., Middleton, P.G., Dorin, J. R., Stevenson, B. J., Gao, X., Durham, S. R., Jefferey, P. K., Hodson, M. E., Coutelle, C., Huang, L., Proteous, D. J., Williamson, R. and Geddes, D. M., Liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat. Med.*, 1, 39-46 (1995).

Carter, B. J., Viral vectors for gene therapy. In: Biotecnology (Vol 5a 2nd Ed.) Mountain A. et al., (eds). *Wiley VCH*, 1999, pp. 395-426.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint-Basile, G., Le Deist, F., Fischer, S., Gene therapy of severe combined immunodeficiencies. *Transfus. Clin. Biol.*, 7(3), 259-260 (2000).

Chang, A. E., Li, Q., Bishop, D. K., Normolle, D. P., Redman, B. D. and Nickoloff, B. J., Immunogenetic therapy of human melanoma utilizing autologous tumor cells transduced to secrete granulocyte-macrophage colony-stimulating factor. *Hum. Gene. Ther.*, 11(6), 839-850 (2000).

Cherng, 1999 Related Articles, Books

Cherng, J. Y., Talsma, H., Crommelin, D. J. and Hennink, W. E., Long term stability of poly((2-dimethylamino)ethyl methacrylate)-based gene delivery systems. *Pharm. Res.*, 6(9), 1417-1423 (1999).

Chirmule, N., Immune responses to adenoviruses and adenoassociated virusesin humans. *Gene Ther.*, 6, 1574-1583 (1999).

Choi, Y. H., *J. Control. Release* 54, 39-48 (1998).

Chung, R. Y. and Chiocca, E. A., Gene therapy for tumors of the central nervous system. *Surg Oncol Clin N Am.*, 7(3), 589-602 (1998).

Chung-Faye, G. A., Kerr, D. J., Young, LS. and Searle, P. F., Gene therapy strategies for colon cancer. *Mol. Med. Today.*, 6, 82-87 (2000).

Clackson, T., Regulated gene expression systems. *Gene Ther.*, 7, 120-125 (2000).

Condreay, J. P., Witherspoon, S. M., Clay, W. C. and Kost, T. A., Transient and stable gene expression in mammalian cells transduced with recombinant baculovirus. *Proc. Natl. Acad. Sci. USA.*, 96, 127-132 (1999).

Costantini, L. C., Jacoby, D. R., Wang, S., Fraefel, C., Breakfield, X.O. and Isacson, O., Gene transfer to the nigrostriatl system by hybrid herpes simplex virus/AAV amplicon vectors. *Human Gene Ther.*, 10, 2481-2494 (1999).

Cristiano, R. J. and Roth, J. A., Epidermal growth factor mediated DNA delivery in to lung cancer cells via the epidermal growth factor receptor. *Cancer Gene Ther.*, 3, 4-10 (1996).

Cruiel, D. T., Pilewski, J. M. and Albelda, S. M., Gene therapy approaches for inherited and acquired lung diseases. *Am. J. Res. Cell. Biol.*, 14, 1-18 (1996).

Curiel, D. T., Pilewski, J. M. and Albelda, S. M., Gene therapy approaches for inherited and acquired lung diseases. *Am. J. Respir. Cell Mol. Biol.*, 14(1), 1-18 (1996).

Dass, C. R., Walker, T. L., Burton, M. A. and Decruz, E. E., Enhanced anticancer therapy mediated by specialized liposomes. *J. Pharm. Pharmacol.*, 49(10), 972-975 (1997).

Datwyler, D. A., Eppenberger, H. M., Koller, D., Bailey, J. E. and Magyar, J. P., Efficient gene delivery in to cardiomyocytes by recombinant Sindibis virus. *J. Mol. Med.*, 77, 859-864 (1999).

Davis, B. R., Brown, J. Y., Prokopishyn, N. L., Luo, Z., Smith, M. R., Wang, J., Carsurd, N. D. V. and Brown, D. B., Glass needle mediated microinjection of macromolecules and transgenes in to primary human blood cell/ progenitor cells. *Blood*, 95(2), 437-444 (2000).

De Smedt, S. C., Demeester, J. and Hennik, W. E., Cationic polymer based gene delivery systems. *Crit. Rev. Thera. Drugs Carrier Sys.*, 17(2), 113-126 (2000).

Dranoff, G., Gene therapy: meeting report. *Biochimica Biophysica Acta*, 1332, 21-24 (1997).

Eastman, S. J., Siegel, C., Tousignant, J., Smith, A. E., Cheng, S. H. and Scheule, R. K., Biophysical characterization of cationic lipid: DNA complexes. *Biochim Biophys Acta.*, 1325(1) 41-62 (1997).

Esposito, E., Sobben, S., Cortesi, R., Menegatti, E. and Nastruzzi, C., Preparation and characterization of cationinc microspheres for gene delivery. *Int. J. Pharmaceutics*, 189, 29-41 (1999).

Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M. and Felgner, P. L., Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* 269(4), 2550-2561 (1994).

Felgner, P., Non-viral strategies for gene therapy. *Sci. Am.*, 276, 86-91 (1997).

Felgner, P. l., Barenholz, Y., Behr, J. P., Cheng, S. H., Cullis, P., Huang, L., Jessee, J. A., Seymour, L., Szoka, F., Thierry, A. R., Wagner, E. and Wu, G., Nomenculature of synthetic gene therapy. *Human Gene Ther.*, 8, 511-512 (1997).

Feltquate, D. M., HeaNEY s., Webster, R. G. and Robinson, H. L., Different T-helper cell types and antibody isotypes generated by saline and gene gun immunization. *J. Immunol.*, 158, 2278-2284 (1997).

Ferrari, S., Pettenazzo, A., Garbati, N., Zacchello, F., Behr, J. P. and Scarpa, M., Polyethylenimine shows properties of interest for cystic fibrosis gene therapy. *Biochim Biophys Acta*, 1447(2-3), 219-225 (1999).

Feuerbach, F. J. and Crystal, R. G., Progress in human gene therapy. *Kidney Int.*, 49, 1791-1794 (1996).

Finsinger, D., Remy, J. S., Erbacher, P., Koch, C. and Plank, C., Protective copolymers for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. *Gene Ther.*, 7(14) 1183-1192 (2000).

Fujimoto, J. and Kaneda, Y., Reversing liver cirrhosis: impact of gene therapy for liver cirrhosis. *Gene Ther.*, (3), 305-306 (1996).

Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C. and Robinson, H. L., DNA vaccines : protective immunization by parenteral, mucosal and gene gun inocculations. *Proc. Natl. Acad. Sci. USA.*, 90, 11478-11482 (1993).

Gao, Z. -G., Choi, H. -G., Shin, H. -J., Park, K. -M., Lim, S.-J.,, Hwang, K. -J. and Kim, C. -K., Physicochemical characterization and evaluation of a microemulsion system for oral delivery of cyclosporin A. *Int. J. Pharm.*, 161, 75-86 (1998).

Garnett, M. C., Gene delivery systems using cationinc polymers. *Crit. Rev. Thera. Drugs Carrier Sys.*, 16(2), 147-207 (1999).

Geddes, D. M. and Beckles, M. A., Gene therapy for alpha 1-antitrypsin deficiency. *Monaldi Arch. Chest. Dis.*, 50 (5), 388-393 (1995).

Glasspool-Malone, J., Somiari, S., Drabick, J. and Malone, R. W., Efficient nonviral cutaneous transfection. *Mol. Therapy*, 2(2) 140-146 (2000).

Gong , J., Chen, D., Kashiwaba, M., Li, Y., Chen, L., Takeuchi, H., Qu, H., Rowse, G. J., Gendler, S.J. and Kufe, D., Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proc. Natl. Acad. Sci.*

USA., 95(11), 6279-6283 (1998).

Grossman, M., Raper, S. E. and Wilson, J. M., Transplantation of genetically modified autologous hepatocytes into nonhuman primates: feasibility and short-term toxicity. *Hum. Gene Ther.*, 3(5), 501-510 (1992).

Guy, A. C. -F., David, J. K., Lawrence, S. Y. and Peter, F. S., Gene therapy strategies for colon cancer. *Mol. Medicine Today*, 6, 82-87 (2000).

Habeck, M., New approaches to treating haemophilia. *Mol. Med. Today,* 6(6) ,214-215 (2000).

Hara, T., Liu, F., Liu, D. and Huang, L., Emusion formulations as a vector for gene delivery in vivo and in vitro. *Adv. Drug Deliv. Res.*, 24, 265-271 (1997).

Hart, S. L., Synthetic vectors for gene therapy. *Expert Opin. Ther. Patents*, 10(2), 199-208 (2000).

Hasan, U. A., Abai, A. M., Harper, D. R., Wren, B. W. and Morrow, W. J. Nucleic acid immunization: concepts and techniques associated with third generation vaccines. *J. Immunol. Methods.,* 229(1-2) 1-22 (1999).

Herweijer, H., Fritz, J. D., Hagstorm, J. E. and Wolff, J. A., Direct gene transfer in vivo. In: Chang PL (ed.) *Somatic gene therapy*, CRC Press, Tokyo, 1995, pp. 183-202.

Hirata, R. K. and Russell, D. W., Design and packaging of adenoassociated virus gene targeting vectors. *J. Virol.*, 74(10), 4612-4620 (2000).

Hogset, A., Prasmickaite, L., Tjelle, T. E. and Berg, K., Photochemical transfection: a new technology for light induced site directed gene delivery. *Human Gene Ther.*, 11(6), 869-880 (2000).

Hughes, M., Vassilakos, A., Andrews, D. W., Hortelano, G., Belmont, J. W. and Chang, P. L., Delivery of secretable adenosine deaminase through microcapsules-a novel approach to somatic gene therapy. *Human Gene Ther.*, 5, 1145 (1994).

Jaroszeski, M. J., Gilbert, R., Nicolau, C. and Heller, R., In vivo gene delivery by electroporation. *Adv. Drug Deliv. Rev.*, 35, 131-137 (1999).

Kabanov, A., Taking polycation gene delivery systems from *in vitro* to *in vivo. P.S.T.T.*, 2(9), 365-372 (1999).

Kabanov, V. A. and Kabanov, A. V., Interpolyelectrolyte and block ionomer complexes for gene delivery: physicochemical aspects. *Adv. Drug Deliv. Rev.*, 30(1-3), 49-60 (1998).

Kaneda, Y., Saeki, Y. and Morishita, R., Gene therapy using HVJ-liposomes: the best of both the worlds? *Mol. Med. Today,* 5, 298 (1999).

Kantoff, P. W., Kohn, D. B., Mitsuya, H., Armentano, D., Sieberg, M., Zwiebel, J. A., Eglitis, M. A., McLachlin, J. R., Wiginton, D. A. and Hutton, J. J., et al. Correction of adenosine deaminase deficiency in cultured human T and B cells by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA.*, 83(17), 6563-6567 (1986).

Karin, N., Gene therapy: the ball takes a spin towards multifactorial diseases. *Israel Med. Assoc. J.*, 2(1), 58-59 (2000).

Kawakami, S., Fumoto, S., Makiya, N., Yamashita, F. and Hashida, N., In vivo gene delivery to the liver using novel galactosylated cationic liposomes. *Pharm. Res.*, 17(3), 306-313 (2000).

Kim, A., Yun, M. -O., Oh, Y. -K., Ahn, W. -S. and Kim, C. -K., Pharmacodynamics of insulin in polyethylene glyol-coated liposomes. *Int. J. Pharm.*, 180, 75-81 (1999).

Kim, C. K. and Jeong, E. J., Enhanced lymph node delivery and immunogenecity of hepatitis B surface antigen entrapped in galactosylated liposomes. *Int. J. Pharm.*, 147, 143-151 (1997).

Kim, C. K., Im, E. -B., Lim, S -J., Oh Y. -K. and Han S.-K., Development of the glucose-triggered pH-sensitive liposome for a potential insulin delivery. *Int. J. Pharm.*, 101, 191-197 (1994).

Kim, C. K., Jeong, E. J. and Kim, M. H., Comparison of in vivo fate and immunogenecity of hepatitis B surface antigen incorporated in cationic neutral liposomes. *J. Microencap.*, 17(3), 297-306 (2000).

Kim, T. W., Chung, H., Kwon, I. C., Sung, H. C. and Jeong, S. Y., In vivo gene transfer to the mouse nasal cavity mucosa using a stable cationic lipid emulsion. *Mol. Cell.* 10(2) 142-147 (2000).

Kreigh, A. M., Direct immunologic activities of CpG DNA and implications for gene therapy. *J. Gene Med.*, 1, 56-63 (1999).

Latchman, D. S., Gene therapy using herpes simplex virus-based vectors. *Histol Histopathol.*, 15(4), 253-259 (2000).

Laurent, N., Wattiaux-De Coninck, S., Mihaylova, E., Leontieva, E., Warnier-Pirotte, M. T., Wattiaux, R. and Jadot, M., Uptake by rat liver and intracellular fate of plasmid DNA complexed with poly-L-lysine or poly-D-lysine. *FEBS Lett.* 443(1) 61-65 (1999).

Ledley, F. D., Non viral gene therapy: the promise of genes as a pharmaceutical product. *Human Gene Ther.*, 6, 1129-1144 (1995).

Ledley, F. D., Pharmaceutical approach to somatic gene therapy. *Pharm. Res.*, 13(11), 1595-1611 (1996).

Lee, E. R., Marshall, J., Siegel, C. S., Jiang, C., Yew, N. S., Nichols, M. R., Nietupski, J. B., Zeigler, R. J., Lane, M. B., Wang, X., Wan, N. C., Harris, D. J., Smith, A. E. and Cheng, S. H., Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lungs. *Human Gene Ther.*, 7, 1701-1717 (1996).

Lee, M. K., Choi, L., Kim, M. -H. and Kim, C. K., Pharmacokinetis and organ distribution of cyclosporin A incorporated in liposome and mixed micelle. *Int. J. Pharm.*, 191, 87-93 (1999).

Lee, R. J. and Huang, L., Lipid vector systems for gene transfer. *Crit. Rev. Ther. Drug Carrier Sys.*, 14(2), 173-206 (1997).

Leitner, W. W., Ying, H. and Restifo, N. P., DNA and RNA based vaccines: principles, progress and prospects. *Vaccines*, 18, 765-777 (2000).

Li, S. and Huang, L., Non-viral gene therapy: promises

and challenges. *Gene Ther.*, 7, 31-34 (2000).
Lim, D. W., Yeom, Y. I. and Park, T. G., Poly(DMAEMA-NVP)-b-PEG-galactose as gene delivery vector for hepatocytes. *Bioconjug. Chem.* 11(5) 688-695 (2000a).
Lim, Y. B., Han, S. O., Kong, H. U., Lee, Y., Park, J. S., Jeong, B. and Kim, S. W., Biodegradable polyester, poly (alph-(4-aminobutyl)-L-glycolic aicd), as a non-toxic gene carrier. *Pharm. Res.*, 17(7) 811-816 (2000b).
Lin, M. T. S., Pulkinen, L., Uitto, J. and Yoon, K., The gene gun: current applications in cutaneous gene therapy. *Int. J. Dermatol.*, 39, 161-170 (2000).
Liu, F., Song, Y. and Liu, D., Hydrodynamics based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.*, 6, 1258-1266 (1999).
Liu, F., Yang, J., Huang, L. and Liu, D., New cationic lipid formulations for gene transfer. *Pharm. Res.*, 13(12) 1856-1860 (1996).
Luo, D. and Saltzman, W. M., Synthetic DNA delivery system. *Nat. Biotech.*, 18(1), 33-37 (2000).
Mahato, R. I., Smith, L. C. and Rolland, A., Pharmaceutical perspective of nonviral gene therapy. *Adv. Genetics*, 41, 95-156 (1999).
Maheshwari, A., Mahato, R. I., McGregor, J., Han, S., Samlowski, W. E., Park, J. S. and Kim, S. W., Soluble biodegradable polymer-based cytokine gene delivery for cancer treaetment. *Mol. Therapy*, 2(2), 121-130 (2000).
Martin, S. G. and Murray, J. C., Gene transfer for human endothelial cells. *Adv. Drug Deliv. Rev.*, 41, 223-233 (2000).
Mastrobatista, E., Koning, G.A. and Storm, G., Immunoliposomes for targeted delivery of antitumour drugs. *Adv. Drug Deliv. Rev.*, 40, 103-127 (1999).
Mathei, C., Van Damme, P. and Meheus, A., Hepatitis B vaccine administration: comparison between jet-gun and syringe and needle. *Vaccine*, 15(4) 402-4 (1997).
Matthews, C., Jenkuns, G., hilfinger, J. and Davidson, B., Poly-L-lysine improves gene transfer with adenovirus formulated in PLGA microspheres. *Gene Ther.*, 6(9) 1558-1564 (1999).
Maurer, N., Mori, A., Palmer, L., Monck, M. A., Mok, K. W. C., Mui, B., Akhong, Q. F. and Cullis, P. R., Lipid based systems for intracellular delivery of genetic drugs. *Mol. Membrane Biol.*, 16, 129-140 (1999).
Mehrara, B. J., Saadeh, P. B., Steinbrech, D. S., Dudziak, M., Spector, J. A., Greenwald, J. A., Gittes, G. K. and Longarker, M. T., Adenovirus mediated gene therapy of osteoblasts in vitro and in vivo. *J. Bone Mine. Res.*, 14, 1290-1301 (1999).
Mitanchez, D., Doiron, B., Chen, R. and Kahn, A., Glucose stimulated genes and prospects for gene therapy for type-I diabetes. *Endocrinol. Rev.*, 18, 520-540 (1997).
Miyoshi, H., Takahashi, M., Gage, F. H. and Verma, I. M., Stable and efficient gene transfer in to the retina using HIV-based lentiviral vector. *Pro. Natl. Acad. Sci. USA.*, 94, 10319-10323 (1997).
Mochizuki, H., Schwartz, J. P., Tanak, K., Brady, R,O. and Reiser, J., High titers HIV type-I based vector systems for gene delivery in to non-dividing cells. *J. Virol.*, 72, 8873-8883 (1998).
Mohammdi, S. and Hawkins, R. E., Efficient transgene regulation from a single tetracycline controlled positive feedback regulatory system. *Gene Ther.*, 5, 76-84 (1998).
Mok, K. W. C., Lam, A. M. I. and Cullis, P. R., Stabilized plasmid-lipid particles: factors influencing plasmid entrapment and transfection properties. *Biochim. Biophys. Acta*, 1419, 137-150 (1999).
Monahan, P. E. and Samulski, R. J., Adeno-Associated viruses: is clinical success on the horizon? *Gene Ther.*, 7(1), 24-30. (2000).
Montaldo, P. G., Pagnan, G., Raffaghello, L., Pastorino, F., Allen, T. M., Kirchmeire, M. and Ponzoni, M., Anti GD2-immunoliposome mediated targeting of I125-MIBG to neuroblastoma and melanoma cells in vitro. *J. Liposoma Res.*, 9(3), 367-385 (1999).
Morris, S., Kelley, C., Howard, A., Li, Z. and Collins, F., The immunogenicity of single and combination DNA vaccines against tuberculosis. *Vaccine*, 18(20), 2155-2163 (2000).
Nabel, E. G., Yang, Z. Y., Muller, D., Chang, A. E., Gao, X., Huang, L., Cho, KJ. and Nabel, G. J., Safety and toxicity of catheter gene delivery to pulmonary vasculature in a patient with metastatic melanoma. *Human Gene Ther.*, 5, 1089-1094 (1994).
Nakanishi, M., Gene introduction in to animal tissue. *Crit. Rev. Thera. Drug Carrier Sys.*, 12(4), 263-310 (1995).
Nanni P, Forni, G. and Lollini, P. L., Cytokine gene therapy: hopes and pitfalls. *Ann. Oncol.*, 10(3) 261-266 (1999).
Natsume, A., Mizuno, M., Ryuke, Y. and Yoshida, J., Cationic liposome conjugation to recombinant adenoviral vectors reduces viral antigenicity. *Jap. J. Cancer Res.*, 91(4), 363-367 (2000).
Nielsen, L. L. and Maneval, D. C., p53 tumor suppressor gene therapy for cancer. *Cancer Gene Therapy*, 5, 52-63 (1998).
Noguchi, A., Furuno, T., Kawaura, C. and Nakanishi, M., Membrane fusion plays an important role in gene transfection mediated by cationic liposomes. *FEBS Lett.*, 433(1-2):169-173 (1998).
Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R. and Wagner, E., The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther.*, 5(10), 425-433 (1998).
Park, K. M. and Kim, C. K., Preparation and evaluation of flurbiprofen-loaded microemulsion for parental delivery. *Int. J. Pharm.*, 181, 173-179 (1999).
Peng, K. W. and Vile, R.., Vector development for cancer gene therapy. *Tumour Targeting*, 4, 3-11 (1999).
Peng, K. W., Strategies for targeting therapeutic gene delivery. *Mol. Med. Today*, 5, 448-453 (1999).
Ponder, K. P., Systemic gene therapy for cardiovascular

disease. *Trends Cardiovasc Med.*, 9(6) 158-162 (1999).
Pouton, C. W. and Seymour, L. W., Key issues in non-viral gene delivery. *Adv. Drug Deliv. Rev.*, 34, 3-19 (1998).
Puls, R. L. and Minchin, R. F., Gene transfer and expression of a non viral polycation based vector in CD4+ cells.
Reynold, P. N., Feng, M. and Cruiel, D. J., Chimeric viral vectors: the best of the both worlds? *Mol. Med. Today*, 1, 25-31 (1999).
Rolland, A. P., From genes to gene medicines: recent advances in non-viral gene delivery. *Crit. Rev. Ther. Drug Carrier Sys.*, 15(2), 143-198 (1998).
Rols, M.P., Delteil, C., Golzio, M., Dumond, P., Cros, S. and Teissie, J., In vivo electrically mediated protein and gene transfer in murine melanoma. *Nat. Biotechnol.*, 16(2), 168-171 (1998).
Romano, G., Pacilio, C., Giordano, A., Gene transfer technology in therapy: current applications and future goals. *Stem Cells*, 17(4), 191-202 (1999).
Ross, P. C. and Hui, S. W., Polyethlene glycol enhances lipoplex-cell association and lipofection. *Biochim. Biophys. Acta*, 1421, 273-283 (1999).
Ross, P. C., Hensen, M. L., Supabphol, R. and Huis, W., Multilamellar cationic liposomes are efficient vectors for in vitro gene transfer in serum. *J. Liposome Res.*, 8(4), 499-520 (1998).
Russell, S. J., Science, medicine, and the future. Gene therapy. *BMJ*, 315(7118), 1289-1292 (1997).
Sakurai, F., Inoue, R., Nishino, Y., Okuda, A., Mastumoto, O., Taga, T., Yamashita, F., Takakura, Y. and Hashida, M., Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intra-cellular trafficking of plasmid/DNA cationic liposome complexes and the subsequent gene expression. *J. Controlled Release*, 66, 255-269 (2000).
Searle, P.F. and Mautner, V., Adenoviral vectors: not to be sneezed at. *Gene Ther.*, 5, 725-727 (1998).
Shangguan, T., Cabral-Lilly, D., Purandare, U., Godin, N., Ahl, P., Janoff, A. and Meers, P., A novel N-acyl phosphatidylethanolamin-containing delivery vehicle for spermine-condensed plasmid DNA. *Gene Ther.*, 7(9) 769-783 (2000).
Simakajornboon, N. and Davis, S. H., New therapies for cystic fibrosis. J. La State. Med. Soc., 150(12), 629-637 (1998).
Simons, J. W. and Mikha, K., Ex vivo gene therapy using cytokine transduced tumor vaccines: molecular and clinical pharmacology. *Semin. Oncol.*, 2(6), 661-676 (1998).
Sternberg, B., Hong, K., Zheng, W., Papahadjopoulos, D., Ultrastructural cgharacterization of cationic liposome DNA complexs showing enhanced stability in serum and high transfection activity in vivo. *Biophysica Biochimica Acta*, 1375, 23-35 (1998).
Sternberg, B., Sorgi FL. and Huang, L.,. New structures in complex formation between DNA and cationic liposomes visualized by freeze fracture electron microscopy. *FEBS Lett.*, 356, 361-366 (1994).
Stone, D., David, A., Bolonganani, F., Lowenstein, P. R. and Castro, M. G.,Viral vectors for gene delivery and gene therapy with in the endocrine system. *J. Endocrinol.*, 164(2), 103-118 (2000).
Tanaka, T., Cao, Y., Folkman, J. and Fine, H. A., Viral vectors targeted antiangiogenic gene therapy utilizing an angiostatin complementary DNA. *Cancer Res.*, 58, 3362-3369 (1998).
Thompson, A. R., Gene therapy for the haemophilias. *Haemophilia*, 6, 115-119 (2000)
Tomlinson, E. and Rolland, A. P., Controllable gene therapy: pharmaceutics of non-viral gene delivery systems. *J. Controlled Release*, 39, 357-372 (1996).
Tseng, W. C., Haselton, F. R. and Giorgio, T. D., Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression. *J. Biol. Chem.*, 272(41), 25641-25647 (1997).
Varley, A. W. and Munford, R. S., Physiologically responsive gene therapy. *Mol. Med. Today*, 4, 445-451 (1998).
Verma, I. and Somia, N., Gene therapy: promises, problems and prospects. *Nature*, 389, 339-342 (1997).
Vile, R. G., Russell, S. J. and Lemoine, N. R., Cancer gene therapy: hard lessons and new courses. *Gene Ther.*, 7, 2-8 (2000).
Vinogradov, S. V., Bronich, T. K. and Kabanov, A. V., *Bioconj. Chem.*, 9, 805-812 (1998).
Vitiello, L., Bockhold, K., Joshi, P. B. and Worton, R. G., Transfection of cultured myoblasts in high serum concentration with DODAC:DOPE liposomes. *Gene Ther.*, 5, 1306-1313 (1998).
Wadman, M., Germline gene therapy must be spared excessive regulations. *Nature*, 392, 317 (1998).
Walsh, J. H., Gene therapy: back to the drawing board. *Gastroenterol.*, 118, 651-652 (2000).
Wang, Y. and Huang, S., Adenovirus technology for gene manipulation and functional studies. *Drug Dev. Rev.*, 5(1), 10-16 (2000).
Watabe, A., Yamaghuchi, T., Kawanishi, T., Uchida, E., Eguchi, A., Mizuguchi, H., Mayumi, T., Nakanishi, M. and Hayakawa, T., Target cell specificity of fusogenic liposomes: membrane fusion mediated macromolecule delivery in to human blood mononuclear cells. *Biophysica Biochimica Acta*, 1416, 339-348 (1999).
Williams, R. S., Johnston, S. A., Riedy, M., Introduction of foreign genes in to tissues of living mice by DNA coated microprojectiles. *Proc. Natl. Acad. Sci. USA*, 88, 2726-2730 (1991).
Wolfert, M. A., Schacht, E. H., Toncheva, V., Ulbrich, K., Nazarova, O. and Seymour, L.W., Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. *Hum. Gene. Ther.* 7(17), 2123-2133 (1996).
Wolff, J. A., Malone, R.W., Williams, P., Chong, W., Acsadi,

G., Jan, A. and Felgner, P. L., Direct gene transfer in to mouse muscle in vivo. *Science*, 247, 1465-1468 (1990).

Wu, N. and Attai, M.M., Production of viral vectors for gene therapy applications. *Curr. Opin. Biotechnol.*, 11, 205-208 (2000).

Yang, K., Clifton, G. L. and Hayes, R. L., Gene therapy for CNS injury: the use of cationic liposomes. *J. Neurotrauma*, 14, 281-297 (1997).

Yang, N. S. and SuN, W. H., Gene gun and other non-viral approaches for cancer gene therapy. *Nat. Med.*, 1, 481-483 (1995).

Yi, S. W., Yune, T. Y., Kim, T. W., Chung, H., Choi, Y. W., Kwon, I. C., Lee, E. B. and Jeong, S. Y., A cationic lipid emulsion/DNA complex as a physically stable and serum-resistant gene delivery system. *Pharm Res.*, 17(3), 314-320 (2000).

Yukai, H. E., Alexie, A. P., Nayak, J. V., Plowey, J., Falo, Jr. LD. and Huang, L., Intravenous injection of naked DNA encoding flt3 ligand dramatically increases the number of dendritic cells and natural killer cells in vivo. *Human Gene Ther.*, 11, 547-554 (2000).

Zou, L. L., Huang, L., Hayes, R. L., Black, C., Qiu, Y. H., Perez-Polo, J. R., Le, W., Clifton, G. L., Liposome mediated NGF gene transfection following neuronal injury: potential therapeutic injury. *Gene Ther.*, 6, 1179-1183 (1999).

Zoulim, F., Trepo, C., New antiviral agents for the therapy of chronic hepatitis B virus infection. *Intervirology*, 42(2-3), 125-144 (1999).

Zuidam, N.J. and Barenholz, Y., Characterization of DNA-lipid complexes commonly used for gene delivery. *Int. J. Pharm.*, 183, 43-46 (1999).