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Review Article

Pharmaceutical Approach to Somatic Gene Therapy

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The pharmaceutical approach to somatic gene therapy is based on consideration of a gene as a chemical entity with specific physical, chemical and colloidal properties. The genes that are required for gene therapy are large molecules (>1 × 10^6 Daltons, >100 nm diameter) with a net negative charge that prevents diffusion through biological barriers such as an intact endothelium, the plasma membrane or the nuclear membrane. New methods for gene therapy are based on increasing knowledge of the pathways by which DNA may be internalized into cells and traffic to the nucleus, pharmaceutical experience with particulate drug delivery systems, and the ability to control gene expression with recombined genetic elements. This article reviews two themes in the development of gene therapies: first, the current approaches involving the administration of cells, viruses and plasmid DNA; second, the emerging pharmaceutical approach to gene therapy based on the pharmaceutical characteristics of DNA itself and methods for advanced drug delivery.

KEY WORDS: gene therapy; DNA; gene delivery; drug development.

INTRODUCTION

The premise of somatic gene therapy is that genes can be used as pharmaceutical products to cause *in vivo* production of therapeutic proteins. Gene therapy represents a new paradigm for therapy of human disease and also for drug delivery. The new therapeutic paradigm is that disease can be treated at a molecular level by restoring defective biological functions or reconstituting homeostatic mechanisms within cells. This is exemplified by the replacement of genetically defective gene functions in inherited disorders such as cystic fibrosis and inborn errors of metabolism. Multifactorial, acquired diseases may also be treated by gene therapy. For example, cell cycle control genes could be used to treat cancer, and immune-modifying cytokines could be used to treat inflammatory disorders.

The new paradigm for drug delivery arises from the ability to engineer the production of therapeutic proteins from cells *in vivo* and to achieve control over the level and location of the therapeutic gene product. Gene therapy can enhance the therapeutic use of proteins by providing improved kinetics and by restricting expression to specific cells within the body. Most importantly, gene therapy can be used to achieve compartmentalization of therapeutic proteins within the cell in locations, such as the nucleus, mitochondria or membranes compartments that are not effectively targeted by extracellular administration of proteins themselves. Gene therapy may also be used to achieve therapeutic effects from low molecular weight chemical entities, such as prostaglandins or NO, whose synthesis may be induced *in vivo* by overexpression of rate-limiting synthetic enzymes.

Gene therapy thus may be indicated for many common diseases in which *in vivo* expression of a protein would meet a clinical need that is unmet by conventional small-molecule and biological products. This requires that products for gene therapy be: (i) as safe, convenient and cost effective as conventional pharmaceuticals, (ii) administered by conventional routes and are cleared from the body with reproducible kinetics, and (iii) accepted by regulatory bodies, reimbursement agencies and patients.

There is growing confidence that gene therapy will provide important pharmaceutical products in the next decade. The initial approaches to gene therapy have exhibited promising pharmacological effects in several animal models of genetic and acquired disease. More than 150 clinical trials are currently underway in the United States and Europe to assess these technologies. These clinical trials have demonstrated that genes can be introduced into patients by several different methods and will express potentially therapeutic gene products (1–6). Nevertheless, significant hurdles remain. Several recent clinical studies failed to demonstrate the expected pharmacological effects (6–10). Moreover, some of the methods that have been proposed for gene therapy have limiting toxicities, are difficult to manufacture and quality control, or are more costly than current therapies. These methods may provide product opportunities for certain genetic and end stage diseases such as cancer and AIDS. Improved methods are required to provide robust therapies for unmet medical needs associated with common diseases.

There are two major themes in current research in gene therapy. The first is the growing preclinical and clinical experience with several prototype approaches for gene therapy. The second theme is the emergence of a new pharmaceutical

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approach to gene therapy based on consideration of a gene as a chemical entity (i.e. a drug molecule) and pharmaceutical experience with particulate drug delivery. This review will address these two themes separately.

CURRENT STATUS: CELLS, VIRUSES, AND PLASMIDS

Three different approaches for gene therapy can be distinguished based on the nature of the material that is administered to the patient: (i) *cell-based* approaches that involve the administration to the patient of genetically engineered cells, (ii) *virus-based* approaches that involve the administration to the patient of genetically engineered, attenuated or defective *viruses*, and (iii) *plasmid-based* approaches that involve administration to the patient of pharmaceutical formulations of DNA molecules themselves. These approaches represent essentially discontinuous technologies that enable distinct clinical opportunities and entail different clinical risks.

Cell-based Gene Therapy

Cell-based therapy is often referred to as *ex-vivo* therapy since genes are introduced into the cells while they are outside of the body. Cell-based approaches involve removing cells from a patient, introducing genes encoding a therapeutic product permanently into these cells *ex vivo*, and returning the cells to the patient by cell transplantation or transfusion. Once introduced into the body, the modified cells are expected to provide expression of the therapeutic gene product for the life of that cell. The first clinical application of this approach was the treatment of Severe Combined Immune Deficiency (SCID), which is due to inherited defects in the enzyme adenosine deaminase (ADA). Several clinical trials have been performed in which peripheral blood lymphocytes or bone marrow progenitor cells were harvested from affected individuals, the normal ADA gene was introduced permanently into the chromosomes of these cells *ex vivo* using retroviral vectors, and the genetically engineered cells were returned to the patient as an autologous transfusion or transplant (9–11). Initial results demonstrated that the ADA gene can be introduced into cells *ex vivo*, that these cells can be safely introduced into patients and will persist for prolonged periods of time, and that low level expression of ADA can be established.

An analogous approach has been used in a pilot study of gene therapy for familial hypercholesterolemia (LDL-receptor deficiency). In this study, hepatocytes were harvested from patients with familial hypercholesterolemia after partial hepatectomy, the normal LDL-receptor gene was introduced into these cells *ex vivo*, and these cells were transplanted into the liver via the portal vein. This procedure led to detectable expression of LDL-receptor in a small fraction of hepatic cells and a measurable effect on cholesterol metabolism (5–6). Also, cell-based methods for introducing genes into bone marrow are being used in clinical trials to treat Gaucher disease (12–14), AIDS (15–16) and to prevent bone marrow toxicity from chemotherapy (17–20) (reviewed 21). Cell-based approaches are also being investigated for the treatment of cancer (22) and arthritis (23).

Cell-based gene therapy presents several theoretical advantages. First, all of the genetic manipulations are performed outside of the body (*ex vivo*) prior to administration of the genetically engineered cell to the patient. This enables purification, characterization, and even pharmacological manipulation (24–25) of the cell prior to introduction of the therapeutic gene. Second, since cell-based approaches involve the stable integration of genes into the chromosomes of the transplanted cell, this method can be used to establish populations of cells capable of expansion, proliferation or differentiation *in vivo*. For example, the introduction of genes into bone marrow progenitor cells gives rise to diverse lineage of cells containing the transgene (26). Finally, cell-based approaches circumvent the difficulties inherent in *in vivo* delivery of genes in favor of transplanting cells that may be directly implanted into tissues and achieve long-term persistence, cells that may be incorporated into heterotopic neo-organs or cells that may actively migrate to appropriate somatic sites.

Several factors limit the clinical potential of cell-based approaches to gene therapy. First, the cultivation, genetic manipulation, quality control and transplantation of autologous cells is expensive relative to the cost of a conventional pharmaceutical or biological product. Second, there is little clinical experience in cellular transplantation of cells other than bone marrow progenitors and, to a lesser extent, epidermis. Most clinical trials of cell-based gene therapy proposed to date involve transplantation of bone marrow cells or mature lymphocytes cells that are known to repopulate the marrow or circulating blood compartment in humans. While transplantation of hepatocytes, pancreatic cells, myoblasts, epidermal cells, neuronal cells, synovial cells and fibroblasts has been demonstrated in animals, these methods are not routinely available in clinical practice.

Virus-based Therapies

The premise of virus-based gene therapy is that viral vectors carrying therapeutic genes may exploit the highly evolved pathways for infection to achieve efficient delivery and expression of therapeutic genes within the body. Virus-based gene therapy involves genetic engineering of attenuated or defective viruses (viral vectors). These viruses are capable of carrying therapeutic genes into cells by the process of infection, but they are not capable of replicating in humans or inducing viral disease. Several different viral vectors have been developed for gene therapy and are now in clinical trials including those derived from murine leukemia viruses (retroviruses), adeno-associated virus (AAV), and adenovirus. Each viral vector system has unique properties as well as different clinical applications and risks.

Retroviral vectors were the first viral vectors to be employed for gene therapy and have been employed in most of the clinical trials to date. Retroviruses are RNA viruses that have the ability to insert their genes permanently into host cell chromosomes after infection. "Defective" retroviral vectors have been developed that are devoid of the genes encoding viral proteins, but retain the ability to infect cells and insert their genes into the chromosomes of the target cell (27). The strategy for constructing defective retroviral vectors is shown in Figure 1-1. Retroviruses will only efficiently infect dividing cells. Thus, retroviruses are most often used to introduce genes into cells *ex vivo* where cell division can be stimulated with growth-promoting media or specific factors. Retroviral vectors can also be directly administered to patients, though the applica-

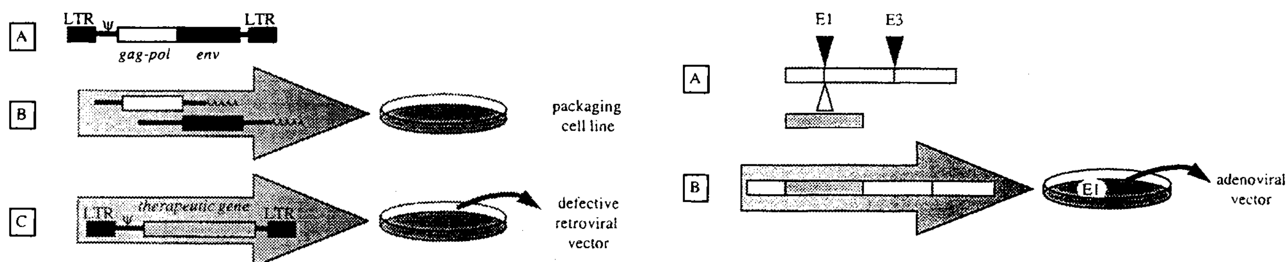


Fig. 1. Construction of viral vectors for gene therapy. (Left) Construction of retroviral vectors. Retroviral vectors currently in clinical trials are derived from the Moloney Murine Leukemia Virus. (A) The retroviral genome consists of a linear strand of RNA that encodes two proteins: the *gag-pol* polyprotein that constitutes the nucleoprotein core of the virus and enzymes required for infection and the *env* protein that constitutes the outer envelope of the virus. The Long Terminal Repeat (LTR) sequences and y sequences are required for packaging genetic material into the virus particle and viral replication. (B) A packaging cell line is produced by introducing genes encoding *gag-pol* and *env* proteins (without the LTR or Y sequences) into cultured cells. These cells produce viral particles that are devoid of viral genetic material. (C) Defective retroviral vectors are produced by combining the therapeutic gene with the LTR and Y sequences and introducing this gene into the packaging cell line. The defective retrovirus produced by these cells contains the therapeutic gene with the LTR and Y sequence within the capsule constituted from the *gag-pol* and *env* proteins. This virus is capable of infecting cells, leading to integration of the therapeutic gene into the chromosome of the target cell; however, it does not express any viral proteins and is incapable of replication. (Right) Construction of adenoviral vectors. Adenoviral vectors are constructed by deleting the E1 sequences from a wild type adenovirus and replacing these viral sequences with sequences encoding a therapeutic gene. The E1 gene is essential for expression of the late proteins of adenovirus that comprise the viral particle. Without the E1 gene, viruses can infect cells but can not replicate. Other genes such as E3 are deleted to minimize the size and immunogenicity of the vector. (B) Adenoviral vectors are produced by introducing the gene encoding the adenoviral vector gene into a cell line that constitutively expresses E1. In the presence of E1 produced by these cells, the late genes within the adenoviral vector are activated resulting in the production of infectious adenoviral vectors. The resulting viral vectors are capable of infecting cells with the therapeutic gene, but since they do not carry the E1 gene, they are not capable of further viral replication.

bility of this approach is limited by the rapid inactivation of retroviruses by human complement. *In vivo* application of retroviruses has been achieved by administration of virus-producing cells directly into tumors. Virus particles released by the producer cell will infect adjacent tumor cells (28). This strategy is being employed in experimental therapies for cancer. Modifications of the retroviral vector have also been described that prevent complement activation and complement-mediated elimination (29–30).

Theoretical concerns have been raised about the safety of retroviral vectors (31–35). One concern is that random insertion of the vector sequence into the host cell chromosome may lead to insertional mutagenesis and oncogenesis. Another concern is that the propensity of RNA viruses to recombine with other viral or cellular RNAs (36–37) will lead to new replication-competent retroviruses (RCR) with unknown properties (38). Recombination of retroviral sequences to form RCR has been described in preclinical studies and during retroviral manufacture (38–39). Clinical studies have not revealed recombination events *in vivo* or any adverse events related to the presence or function of the retroviral vector.

Adeno-associated virus (AAV) is a DNA virus that is capable of permanently inserting its genome into the chromosomes of the host cell (40). As a DNA virus, however, AAV may be less susceptible to recombination than retroviruses. Moreover, wild-type AAV is capable of infecting both dividing and non-dividing cells and inserting its genome into specific sites in the host cell chromosome. AAV vectors can be constructed that are completely defective and retain the ability to integrate genes into host cell chromosomes. Site-specific recombination, however, is not observed with defective AAV vectors. AAV vectors have been shown to infect some nondividing cells (41), though recent data suggests that AAV vectors preferentially infect cells in S phase during the replicative cycle (42). The major limitation of AAV has been difficulty in developing packaging cell lines that will produce sufficient titers of the virus for clinical use without the presence of helper virus (43–44). The first clinical trial using an AAV vector for treatment of cystic fibrosis has recently commenced (45).

Adenoviral vectors are designed to be administered directly to patients. These vectors will infect dividing and non-dividing cells in many different tissues *in vivo* including airway epithelial cell, endothelial cells, hepatocytes and various tumors (46). Because of the high efficiency of adenoviral infection *in vitro* and *in vivo*, adenoviral gene transfer is considered the benchmark against which other delivery methods are compared.

Unlike retroviral and AAV vectors, adenoviral vectors do not integrate into the chromosome of the host cell. Rather, genes introduced into cells using adenoviral vectors are maintained in the nucleus as an extrachromosomal element (episome) and will provide expression of the therapeutic gene for a finite period of time until the gene is eliminated. Adenoviral vectors are currently being employed in clinical trials to treat cystic fibrosis (4, 7) and various cancers (47–51).

The major limitation of adenoviral vectors is their demonstrated toxicity. Adenoviral vectors are attenuated (not defective) viruses that express several viral proteins. The strategy for constructing adenoviral vectors is shown in Figure 1-2. While the attenuated vector is not capable of effective replication, it does induce cytopathic and immunogenic responses *in vivo*. Preclinical and clinical studies have demonstrated that the level and persistence of gene expression may be inhibited by the immunological responses against the adenoviral particle and inflammation in tissues targeted by the vector. In the first controlled clinical trial using adenoviral vectors, the inflammatory response to the vector prevented administration of an effective dose of the therapy (7). Moreover, the humoral immune response elicited by *in vivo* administration of an adenoviral

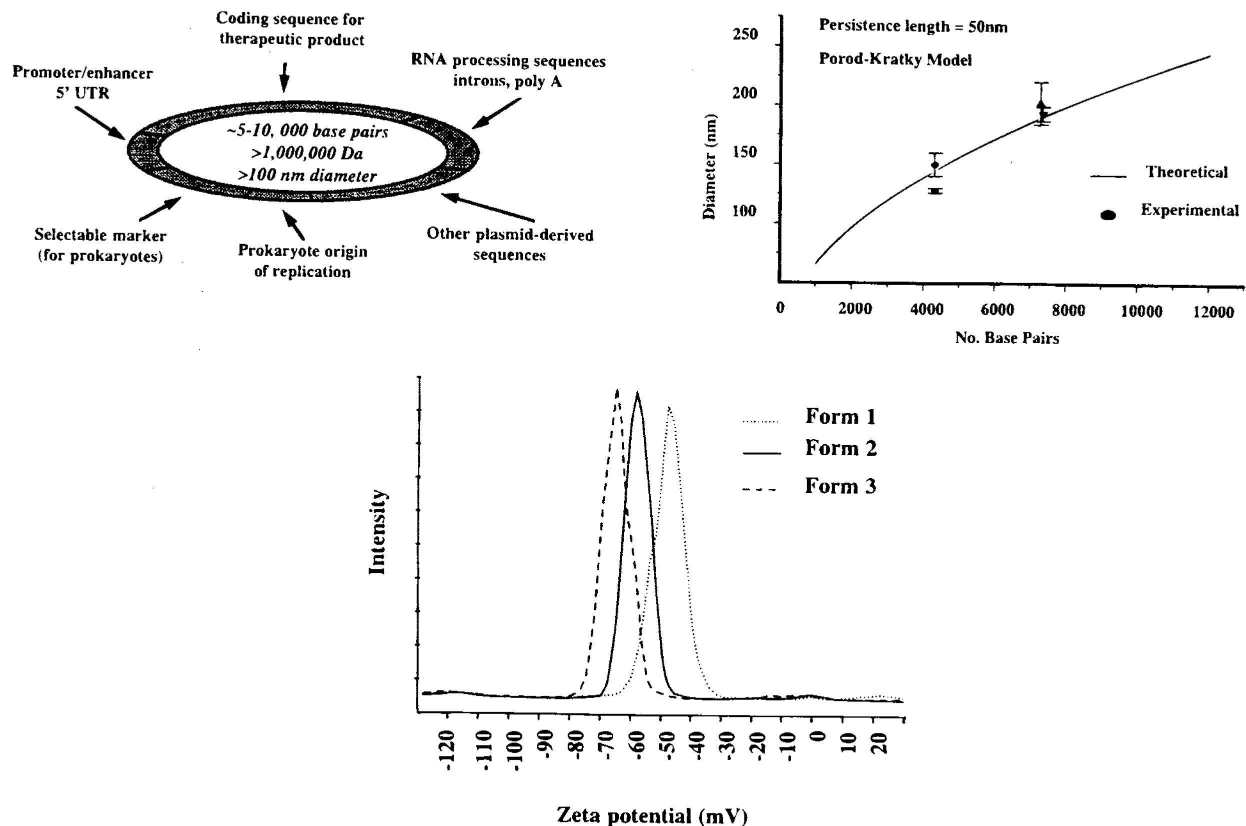


Fig. 2. Physical characteristics of plasmid DNA. (A) For gene therapy, plasmids are constructed containing a sequence encoding a therapeutic gene, sequences for expression in mammalian cells and sequences for cloning in bacteria. Such a recombinant plasmid is 5–10,000 base pairs in length and has a molecular weight >1,000,000 Da. (B) Hydrodynamic diameter of plasmid DNA of different length calculated by the Porod-Kratky Model and determined experimentally. (*Data from ref 179*) (C) Zeta potential of different conformations of plasmid DNA measured by Doppler electrophoretic light scattering (1:supercoiled; 2:relaxed; 3:linear). (*Data from ref 227*).

vector includes blocking antibodies that prevent effective repeat administration of the adenoviral vector (52). A cytotoxic T lymphocyte (CTL) response is also induced against cells infected with the adenoviral vector (53, 54–55). This CTL response limits the duration of the therapeutic effect by selectively eliminating cells that are transduced with the adenoviral vector (56–57). It has been shown that the duration of expression from an adenoviral vector is longer in nude mice (57) and in animals treated with immunosuppressants to induce tolerance (58–60) than in normal, immunocompetent animals. An additional concern is that 13% of normal individuals and 21% of the patients with cystic fibrosis have E1a in their epithelial cells as a result of routine infection with wild-type adenovirus (61). E1a in these cells could allow replication of the attenuated adenoviral vectors *in vivo*.

Current research on adenoviral vectors is focused on reducing their immunogenicity by incorporation of additional mutations to attenuate further expression of immunogenic viral proteins (62), increasing the potency of adenovirus to minimize the immune challenge, and coadministering immunosuppressive regiments with the adenoviral vector therapy (58–60). While immunosuppression has been effective in animal models, the challenge of safely eliminating the immunological response to viral infection remains formidable. Furthermore, the need for immunosuppression in conjunction with adenoviral vectors would limit the applicability of this approach to treating certain severe diseases.

Plasmid-based Therapies

Plasmid-based approaches to gene therapy, often termed "non-viral," involve administration of purified DNA or formulations of DNA directly to patients (63). As described in more detail below, studies have demonstrated that this DNA can be taken up by cells within the body and can direct expression of recombinant proteins. For example, intramuscular administration of DNA has been shown to produce gene expression in mature muscle cells (64), and these methods are being employed in clinical trials for gene vaccines (65).

The most extensively studied approach for plasmid-based therapy involves the delivery of DNA with lipids. The prototype cationic lipid-based gene delivery system contains DOTMA (1,2-dioleyloxypropyl-3-trimethyl ammonium bromide) and DOPE (dioleoylphosphatidylethanolamine). Formulations with DOTMA have been shown to provide expression in pulmonary epithelial cells in animal models (66–67) and are currently being employed in a clinical trial for replacement of α-antitrypsin in patients with a genetic deficiency of this protein. Other formulations containing analogous cationic lipids such as DC-CHOL (3 [N-(N′,N′-dimethylaminoethane)carbamoyl]cholest-

erol), DOTAP (1,2,-dioleoyloxy-3-(trimethylammonio)propane) and DMRIE (1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide) are being used in clinical trials to treat cystic fibrosis (3, 68) or cancer (2, 69).

There are several theoretical advantages to non-viral therapies including their apparent safety and cost of manufacture. No significant toxicity has been encountered in preclinical toxicology studies using several cationic lipid:DNA complexes (70–74) or in initial human studies (2, 75–76). Significantly, despite a theoretical concern about raising anti-DNA antibodies (77), there is no evidence for the formation of antibodies against DNA or antinuclear antibodies after administration of "naked DNA" or DNA formulated with lipids or proteins (2, 74, 76–78). The major limitation of plasmid-based approaches has been that both the efficiency of gene delivery to several important somatic targets (e.g. liver and lung) and the level of gene expression that can be achieved *in vivo* is lower using non-viral approaches than adenoviral vectors.

CURRENT STATUS: SUMMARY

Several generalizations can be made concerning current approaches to gene therapy. First, *cell-based* approaches are limited to those clinical situations where cell transplantation technologies are clinically established, where the stable incorporation of the therapeutic gene into the chromosomes of the cell is clinically advantageous, and where the cost of *ex vivo* genetic manipulations of cells would not increase the current cost of clinical care. An ideal target for cell-based therapy is the hematopoietic stem cell of the bone marrow since bone marrow transplantation is established in clinical practice. Stable integration of genes into stem cells is required to achieve the proper distribution of the therapeutic gene in the mature progeny of these cells, and the cost of genetic manipulation of bone marrow may not add significantly to the current cost of autologous or allogenic bone marrow transplantation.

Second, the early focus of gene therapy on *virus-based* approaches with experimentally demonstrated toxicities led to the perception that *in vivo* gene therapy, in general, might be applicable only to life-threatening end stage disorders. While viral therapies show considerable promise in many preclinical models, their limited therapeutic index and immunological responses to viral vectors may limit their application to selected end-stage or otherwise untreatable disorders.

Third, while *plasmid-based* approaches offer a favorable safety profile, these approaches do not yet provide sufficiently robust levels of gene expression to achieve pharmacological effects for many potentially important clinical indications. Current technologies are applicable to biological targets where the effects of low level gene expression are amplified such as expression of a hormone or growth factor for local or systemic effects, expression of an antigen or cytokine to elicit a systemic immune response, or expression of enzymes capable of producing biologically active metabolites.

It is important to recognize that these approaches to gene therapy employ methods for cell transplantation, molecular virology and *in vitro* transfection that have not been optimized for *in vivo* gene therapy. Moreover, these methods have not been optimized for pharmaceutical applications. While incremental improvements in these methods may lead to products for certain clinical indications, these technologies are unlikely to fulfill the broad promise of gene therapy as an approach for treating many common diseases. What is required is a first-principle approach to developing genes as pharmaceutical products.

The Pharmaceutical Approach to Gene Therapy

The emerging pharmaceutical approach to gene therapy is based on the consideration of a gene as a chemical entity with specific physical, chemical and colloidal properties as well as increasing knowledge of the pathways by which particulate materials, such as DNA, may be internalized into cells and traffic through different cellular compartments. This approach is aimed at exploring and exploiting the pharmaceutical characteristics of genes and the biological characteristics of target tissues to achieve controlled gene delivery and gene expression. This is achieved by developing (i) advanced gene delivery systems that are based on the principles of drug delivery and (ii) controlled gene expression systems based on an understanding of transcription and translation control mechanisms of the cell. Pharmaceutical experience with the delivery of particulate materials (79) including, but not restricted to liposomes, is particularly important since this science provides an understanding of how the properties of a particulate material such as its size, charge, and surface characteristics determine its distribution and behavior within the body. It should be emphasized that the pharmaceutical approach to gene therapy is not aimed at constructing a synthetic virus to mimic the pathways of viral infection. The initial emphasis on viral infection as a paradigm for gene delivery has perhaps limited consideration of genes as chemical entities. The remainder of this review focuses on current understandings of the pharmaceutical properties of DNA and approaches for gene delivery using pharmaceutical delivery systems.

THE PHARMACEUTICAL PROPERTIES OF DNA

Effective gene therapy requires that a gene encoding a therapeutic product be administered, delivered to the nucleus of the target cell and expressed to produce a gene product. This requires that the gene achieve access to the target cell, uptake and internalization into the cell, and trafficking through the body of the cell across the nuclear membrane and into the nucleus. Once within the nucleus, DNA must be transcribed into mRNA, the mRNA must be processed and translated, and the resulting protein must be post-translationally modified to produce the therapeutic gene product.

Physical Properties of Plasmid DNA

The fundamental challenge of gene therapy is that a DNA molecule encoding a therapeutic protein is a charged, colloidal material that does not effectively cross biological barriers such as an intact endothelium, the plasma or nuclear membrane. For gene therapy, the gene sequence encoding the therapeutic protein is commonly recombined in a closed circular piece of DNA termed a plasmid that contains sequences that allow the gene to be grown in bacteria including a prokaryotic origin of replication and selectable marker. Within the plasmid, the gene encoding the protein is also combined with sequences that direct expression of the protein in mammalian cells. These sequences include promoters, enhancers, introns, 5′URTs, 3′UTRs, and polyadenylation sequences that direct transcription and pro-

cessing of the mRNA as well as the translation of the mRNA and post-translational processing of the protein (Figure 2-1).

A plasmid containing these sequences comprises a molecule ~5–10,000 base pairs in length with a molecular weight >1 million daltons and a hydrodynamic diameter in aqueous suspension of >100 nm (figure 2-2). Plasmids may exist in three tertiary structures. Form I is a supercoiled molecule. Form II an open circle formed by nicking one strand of the DNA which relaxes the torsional stress on the supercoiled plasmid. Form III a linear molecule formed by breaking the double stranded plasmid sequence. DNA has a high negative surface charge due to the phosphate of each nucleotide, and the ζ-potential of different forms of plasmid DNA range from −30 to −50 mV in an aqueous colloidal suspension (Figure 2-3) (80).

DNA within the body is located almost exclusively in the nucleus where it is tightly complexed with nuclear proteins in quaternary structures termed nucleosomes. DNA participates in few chemical reactions within the body and is modified primarily by sequence-specific and non-specific binding to proteins and enzymatic reactions. DNA in mammalian cells is modified by sequence-specific methylation at C-G sequences and is degraded and repaired by various endonucleases, exonucleases and polymerases.

Condensation of Plasmid DNA

The genomes of many viruses are large (10,000–>1,000,000 bases) yet can be accommodated within a viral particle of <100 nm. This is achieved by physical condensation of DNA within the viral capsid to occupy a volume that is 10^4–10^6 times less volume than that of uncondensed DNA. In order for DNA to condense into a compact toroid or sphere, unfavorable free energies associated with DNA bending, entropy of mixing, and electrostatic repulsion forces must be overcome. The major resistance toward DNA condensation arises from electrostatic repulsion between the array of negatively charged phosphates on the polyanion. The unfavorable free energy electrostatic repulsion can be 1–3 orders of magnitude more than all other energy barriers combined (81–82).

Electrostatic barriers to DNA condensation can be overcome through the use of many different multivalent organic or inorganic cations (81–83). Under conditions approaching infinite dilution, these polycations are capable of packaging DNA into particulate systems that have a hydrodynamic radius ~35 nm.

Pharmacokinetics and Pharmacodynamics of DNA

DNA administered into the body by interstitial, intravenous or inhalation administration is rapidly eliminated from the site of administration. Rapid degradation of DNA has been observed after interstitial injection into skeletal muscle (84), thyroid (85), joints (86), and liver (87), and after instillation into the airways (88). After intravascular administration to mice, plasmid DNA (89) or linear DNA fragments (90) are rapidly eliminated both through degradation within the blood compartment as well as

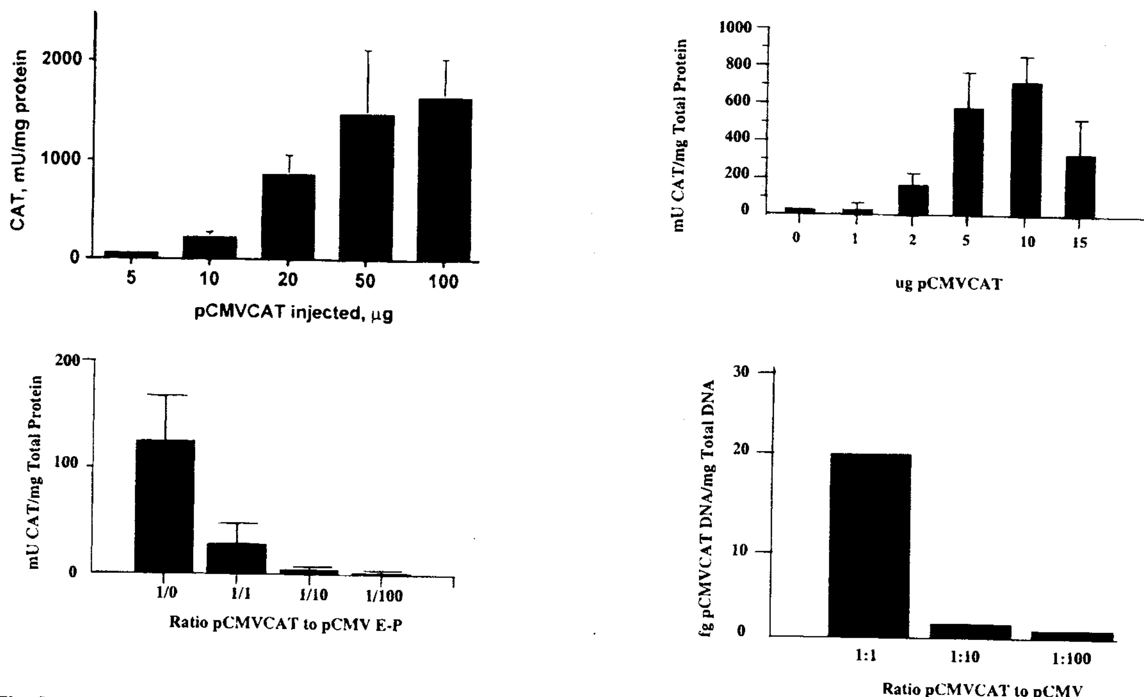


Fig. 3. Saturable kinetics of DNA uptake and expression. (A) Expression of reporter gene in muscle after intramuscular injection as a function of the amount of DNA administered. These expression system in these experiments contains the CMV immediate early promoter and enhancer. (*Data from ref 100*) (B) Expression of reporter gene in thyroid after intra-thyroid injection as a function of the amount of DNA administered. The expression system in these experiments contains the CMV immediate early promoter and enhancer. (*Data from Sikes et al., in preparation*) (C) Competitive inhibition of reporter gene expression in thyroid by coadministration of plasmids containing of CMV promoter. (*Data from Sikes et al., in preparation*) (D) Competitive inhibition of nuclear localization in thyroid by coadministration of plasmids containing the CMV promoter (*Sikes et al., in preparation*). For competition experiments in 3.3 and 3.4, thyroids were injected with 4 ug of DNA and 396 ug of plasmids containing differing ratios of the CMV promoter or unrelated sequences.

by clearance from the blood compartment by cells of the mononuclear phagocyte system in the spleen and liver (Kupffer cells). The degradation of plasmid DNA within tissues or within the blood compartment results from the action of both endonucleases and exonucleases. This can be replicated *in vitro* using tissue extracts, blood or plasma (89, A. Rolland *et al.*, personal communication).

The clearance of plasmid DNA by the liver is a receptor-mediated process (89, 90) that can be inhibited by coadministration of compounds known to compete for binding to the scavenger receptor on Kupffer cells (89). Clearance by this receptor is highly efficient, and the rate of clearance quantitatively approaches the fraction of hepatic blood flow (89). Once bound to the receptor, DNA is slowly internalized into cells and can be released from the receptor by perfusion of animals with DNAase for up to one hour after administration (90). Studies in primates using linear DNA fragments similarly demonstrate that the liver is the major site of clearance of intravenously administered DNA (91). DNA binding to the kidney has also been observed after injection into the renal artery (92); however, little DNA is seen in the kidney after intravenous administration due to rapid clearance by the liver.

In Vivo Function of Administered DNA

It has long been observed that the injection of purified viral DNA or DNA precipitates into the bloodstream of animals will lead to production of infectious viral particles, suggesting that some cells within the body are capable of taking up and expressing these sequences (93). Studies also demonstrate that intramuscular administration of purified ("naked") plasmid DNA leads to the uptake and expression of the injected genes in mature skeletal muscle cells (64, 84, 93–100) as well as cardiac muscle cells (101–104) of various species. The uptake of plasmid DNA by muscle cells is relatively inefficient, producing detectable gene expression in rodents in only 10^2–10^3 cells/muscle adjacent to the track of injection (84, 96). The level of expression is saturable as a function of the dose of DNA administered (Figure 3-1). The efficiency and reproducibility of DNA uptake and expression in muscle can be improved by preinjecting tissues with hypertonic (20%) sucrose (97), which may enhance the dispersion of the injected DNA within the muscle tissue.

Several lines of evidence suggest that the mature myotube is the target for DNA uptake after intramuscular administration. Immunohistochemical studies demonstrate the localization of recombinant gene products within mature myotubes. In addition, gene products can be produced at high levels using promoters that are specific for mature muscle cells, such as the α-skeletal actin promoter (Figure 4-2) (Eastman *et al.*, unpublished data) further suggesting that the mature myotube is the target for DNA uptake after intramuscular administration. In contrast, however, it has been observed that the delivery or expression of plasmid DNA can be enhanced by treatments that are toxic to muscle and increase the fraction of immature myoblasts (105) such as bupivicaine (106) or snake venom cardiotoxin (97). These data suggest that immature cells are also capable of taking up and expressing gene products. In fact, the observed immunohistochemical staining and promoter activity in mature cells could result from gene delivery to immature cells with subsequent fusion into myotubes.

One of the most striking observations from studies with intramuscular administration of plasmid DNA is that gene expression can persist for many months (95, 96, 102, 104). Significantly, there is evidence that this DNA remains resident in the cell for extended periods of time without integration into host cell chromosomes or repair. It is not known whether the persistent expression reflects the continuous presence of DNA in the nucleus, sequential uptake from other cellular compartments, or intracellular mechanisms that may protect DNA against degradation. It has been suggested that the latent (non-replicative) state of the myotube could lead to the long persistence observed after introduction of DNA into these cells, though similar persistence is not seen in other latent cells such as the hepatocyte.

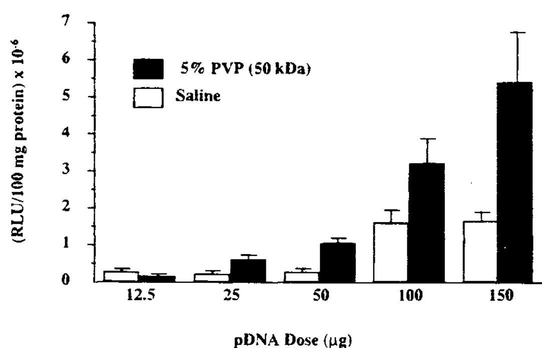
Uptake and expression of DNA has also been observed after direct injection of plasmid into the thyroid (85) or synovium (86) at levels comparable to those observed in muscle. Lower levels of gene expression have been observed after interstitial injection into liver (87), skin (115), instillation into the airways (88), application to the endothelium (107, 108), and after intravenous administration (109). Taken together, these observations demonstrate that there are natural pathways by which exogenously administered DNA can be taken into cells and travel to the nucleus where gene expression can occur.

Mechanisms for Uptake of DNA *In Vivo*

The mechanism of DNA uptake into muscle cells after intramuscular administration has been studied *in vitro* using fluorescent DNA-binding probes and electron microscopic localization of biotinylated DNA. These studies suggest that DNA is taken up by muscle through the T-tubule system and caveolae via potocytosis (94). Potocytosis involves invagination of a caveolae-rich 50 nm diameter vesicles (caveolae) from the cell surface. These light vesicles can be separated in a Percoll gradient as a single plasma membrane fraction (110), which does not disengage from the cell surface of epithelial cells (100). This pathway has been shown to be involved in the transport of folate (100) and other small molecules and in the sequestration of small signalling molecules including inositol phosphoglyceride (111). The uptake of intact plasmid DNA through caveolae in muscle represents the largest molecule demonstrated to be transported by this mechanism.

The mechanism of uptake of DNA into thyroid follicular cells has been studied *in vivo* after direct interstitial injection into this tissue (Sikes *et al.*, in preparation). In thyroid, the injected plasmid DNA localizes within the light lysosomal/endosomal subcellular fractions (1.05–1.06 g/ml) on Percoll gradients, a fraction consistent with the endosomal compartment. The DNA in this fraction is resistant to DNAase I digestion, indicating it is enclosed within the lipid vesicles of those fractions (Sikes *et al.*, in preparation). This endosomal fraction can be clearly differentiated from the plasma membrane-associated subcellular fraction that contains the potosome (112), suggesting that the mechanism of uptake in thyroid differs from that in muscle (Sikes *et al.*, in preparation). The endosomal pathway for the uptake of DNA is similar to that described for *in vitro* uptake of DNA and DNA precipitates (113–114).

Little is known about how DNA escapes from the endosomal or potosomal compartment, moves within the cell, or is taken into the nucleus. The endosome is rich in nucleases,



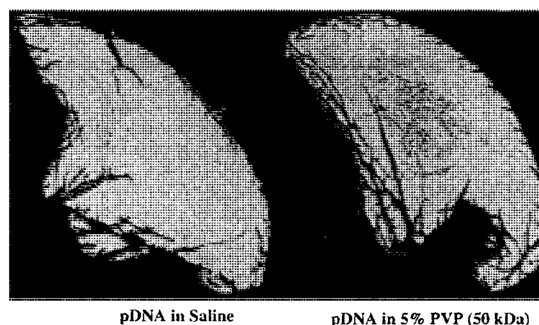


Fig. 4. Enhanced gene delivery to muscle using interactive polymers. (A) Immunohistochemical staining of rat tibialis muscle for −gal at 7 days after I.M. injection of DNA in: (A) saline and (B) 5% PVP (50 kDa). CMV-−gal injected per muscle was 150 μg. The scale shown is in millimeters. (*Data from ref 126*) (B) −gal expression in rat tibialis muscle after I.M. injection as a function of the amount of DNA injected. DNA is injected in formulations comprising saline () or 5% PVP (50 kDa) in 150 mM NaCl. (). (*Data from ref 126*).

and the acidity of this compartment coupled with this enzyme activity would be expected to rapidly degrade DNA. It has also been demonstrated that the internal cytoskeletal network of cells prohibits passive diffusion of molecules larger than 10 nm, thus little passive diffusion of DNA through the cell would be expected (117). Moreover, little passive diffusion of DNA into the nucleus would be expected through the nuclear pore which has a size exclusion of 24 nm. These expectations were confirmed by experiments with microinjection of DNA into the cytoplasm of mature myotubes which demonstrated that DNA did not diffuse effectively through the cell and that the efficiency of cell transformation (e.g., number of cells expressing a reporter gene) after microinjection was inversely related to the distance of the nuclei from the injection site (116).

Several lines of evidence suggest that the trafficking of DNA through the cell and entry into the nucleus may be an active process. Small molecules <9 nm in diameter can pass into the nucleus through the nuclear pore complex (NPC) by passive diffusion (117). The translocation of large molecules including proteins and nucleic acids across the nuclear membrane is known to be an active, energy requiring, signal dependent, saturable and carrier-mediated process (117, 118). These active transport mechanisms have been shown to transport particles as large as 25 nm in diameter through the NPC. This process involves at least four soluble factors including importin α, importin β, RAN (GTPase) and pp15. The nuclear localizing sequence present on many nuclear-targeted proteins is a ligand for the importin α-β-heterodimer. Sequences within importin then bind to the NPC and induce internalization into the nucleus, a process that involves the action of the Ran GTPase and pp15 component of the NPC (117). There is little data on the transport of nucleic acids into the nucleus, though there is considerable data on the mechanism of egrees of newly transcribed RNA. Newly synthesized pre-snRNAs will not passively diffuse through the nuclear membrane or nuclear pore. Egress of these molecules from the nucleus is dependent upon a specific nuclear membrane binding protein RCC1 (117).

There are several mechanisms by which DNA could theoretically enter the nucleus. The nucleocapsids of many viruses contain nuclear targeting sequences and may actively direct nucleic acids through the NPC through a receptor-mediated process. Other viruses that require cell replication for infection may be able to introduce their genomes into the nucleus only during the period of the cell cycle during which the nuclear membrane is dissociated. It has been demonstrated that DNA microinjected into the cytoplasm was rapidly complexed with cytoplasmic proteins and that nuclear uptake can be inhibited by wheat germ agglutinin (WGA) (116). These data suggest that uptake into the nucleus takes place through the nuclear pore complex by a facilitated, energy-dependent process. The mediators of this process are not known.

Kinetic evidence for an active, saturable and potentially receptor-mediated step in nuclear uptake of DNA arises from studies of gene delivery into thyroid follicular cells after interstitial injection into the thyroid. These studies demonstrated that the level of expression from a reporter gene transcribed from a CMV-promoter is saturable as a function of increasing amounts of DNA administered (Figure 3-2). Moreover, expression from this plasmid can be competitively inhibited by coadministration of other plasmids containing the CMV-promoter or fragments of this promoter (Figure 3-3), but not by DNA lacking this sequence or other negatively charged nanoparticles (data not shown). Cell fractionization studies demonstrate that the localization of the CMV-plasmid in the nuclear fraction can be similarly inhibited by coadministration of the same DNA sequences containing the CMV promoter (Figure 3-4), but not by other DNA sequences or particulate materials. This suggests that nuclear uptake of DNA by these cells is a specific, saturable and rate limiting process. This is consistent with the expectation that nuclear uptake would not occur by passive diffusion, but would require an active uptake process. These studies suggest that the mechanism for the uptake of DNA into the nucleus of these cells involves a sequence-specific interaction of the CMV promoter and enhancer sequences on the plasmid with transcription factors located within the cytoplasm. This interaction then leads to nuclear import of the protein:DNA complex through the nuclear pore complex mediated by the nuclear localization sequences contained within these proteins (Sikes *et al.*, in preparation). Band shift experiments demonstrate that there is rapid interaction of plasmid DNA with cytoplasmic proteins, though the identity of these proteins and their role in nuclear uptake or compartmentalization of the plasmid is not known.

Kinetic studies suggest that there are significant differences in the pathways for uptake of DNA into different cells. In

muscle, while gene expression is similarly saturable as a function of the amount of DNA administered (Figure 3-1) (84, 100, 104, 117), delivery or expression can be competitively inhibited by salmon sperm DNA and dextran sulfate (100) compounds that do not inhibit uptake or expression in thyroid follicular cells. In the airway epithelium after topical instillation of plasmid DNA, expression is inhibited by dextran sulfate but not by salmon sperm DNA (88). These data suggest that the pathways for uptake or expression of plasmids in these tissues are different or have distinct kinetic properties. This prediction is consistent with cell biology experiments demonstrating that there are several different potential pathways for DNA uptake (e.g., endosome and potosome). The observation of cell-specific differences in the kinetics of DNA uptake or expression is significant since it implies that different strategies will be necessary to enhance the level of uptake or expression in different tissues. For example, if nuclear uptake is the rate limiting step in gene uptake, then enhancing cell surface interactions or endosomal release would not be expected to increase the net level of gene expression.

Integration

Following *in vitro* transfection of DNA into cells, the administered DNA will become stably integrated into the chromosomes of a small number of cells ($1{:}10^4$–$1{:}10^5$). The mechanism by which DNA is inserted into the host cell chromosome is thought to involve the chromosomal repair mechanisms (119). Despite the demonstrated ability of DNA to integrate into cells *in vitro*, however, there is no reported evidence for integration of DNA into host cell chromosomes after *in vivo* administration. Rather, DNA is maintained as an episome, and the transfected sequences are gradually eliminated from the cell presumably by the action of a nuclease. Significantly, DNA within the target tissue retains the bacterial pattern of methylation, suggesting that these sequences are not repaired or replicated by mammalian enzymes. The quaternary association of this DNA with nuclear proteins has not yet been investigated.

PHARMACEUTICAL METHODS FOR GENE DELIVERY

Studies of DNA as a chemical entity have only begun to systematically describe the dynamics of DNA in the extracellular and intracellular compartments. Already several patterns have emerged. First, plasmid DNA exhibits kinetic and dynamic properties similar to other charged, particulate materials including rapid clearance from the circulation by cells of the reticuloendothelial system, limited diffusion in interstitial or intracellular spaces, and the inability to penetrate the periplasmic or nuclear membranes effectively except by active processes. Second, specific barriers to the *in vivo* delivery of DNA can be identified. These include: (i) the rapid degradation of DNA within tissues or blood by nucleases; (ii) the limited dispersion of DNA from the site of interstitial administration; (iii) the inability of DNA to cross intact basement membranes of the endothelium or epithelium effectively; (iv) the rapid clearance of DNA from the vascular compartment by cells of the reticuloendothelial system; (v) the need for effective interaction with the surface of the target cell to induce internalization; (vi) destruction of DNA in the endosomal/lysosomal compartments by nuclease, acid and/or reducing agents; and (vii) the need to penetrate to the nucleus of cells across the periplasmic membrane and nuclear membrane. Third, the effectiveness of gene delivery *in vivo* is poorly predicted by *in vitro* results. There are many reasons why *in vitro* results would not be recapitulated *in vivo* (Ledley, 1995) including the various biological barriers that are not reflected in *in vitro* models and the interactions between DNA or formulated DNA complexes with serum and blood elements. In fact, many methods for *in vitro* gene transfer are not effective in systems that include high concentrations of serum. Fourth, there are several different pathways by which plasmid DNA may enter cells, and the rate limiting barriers to effective gene delivery differ in different targets. It is not yet possible to describe in detail the complete pathway by which plasmid DNA is taken into a cell after administration.

Because this understanding is incomplete, current efforts are focused primarily on overcoming the well-described initial barriers to gene delivery including the bioavailability of DNA to the selected targets, internalization into the target cell, and egress into the body of the cell from the endosome or potosome. These efforts include the development of devices for gene therapy as well as gene delivery systems and formulations comprising various polymers, lipids, proteins and peptides that may be used to stabilize DNA, control its distribution within the body and induce uptake by target cells. Further understanding of the pathways by which DNA traffics within the cell will reveal new avenues for enhancing gene delivery to specific somatic targets are likely to be recognized.

Devices for Enhancing Bioavailability

Various devices have been developed for enhancing the bioavailability of DNA to the target cell. A simple approach is to contact the target cell physically with catheters or implantable materials containing DNA (107). For example, gene transfer to vascular endothelial cells has been achieved using catheters coated with hydrogel impregnated with DNA. The coated catheters are introduced into a blood vessel to contact the vascular endothelial surface (108).

The bioavailability of DNA after interstitial administration may also be enhanced using needle-free injection devices (jet injection) (120–122). Jet injection into tissues is achieved by projecting a column of liquid directly into the target tissue under high pressure. The column of liquid penetrates superficial layers of tissue becomes dispersed within the tissue. The greater dispersion of the injected material within the tissue increases the number of cells that are available to take up DNA. It has been demonstrated that DNA can be administered by jet injection without damaging the plasmid. Also, this method has been used to achieve increased levels of gene expression in tissues including muscle and epidermis (120, 97, 212–122).

Another device for gene delivery is the "gene gun" or Biolistic™, a ballistic device that projects DNA-coated microparticles directly into the nucleus of cells *in vivo*. Once within the nucleus, the DNA dissolves from the gold or tungsten microparticle and can be expressed by the target cell. This method has been used effectively to transfer genes directly into the skin, liver and muscle (123–125). The major limitation of this approach is that the DNA-coated microparticles are only able to penetrate several millimeters into the target tissue, and

thus effective gene delivery can be achieved only in the superficial layers of most organs.

Interactive Polymers

Formulations of plasmid DNA for intramuscular administration with polymers such as polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) have been explored as a means for enhancing the stability, retention, and dispersion of DNA within muscle (126–127). These polymers are known to enhance the dispersion, retention, and release of conventional drugs (128–129) and are used in several current pharmaceutical products.

Studies have demonstrated that intramuscular injection of plasmid DNA formulated with 5% PVP (50,000 kDa) increases the level of reporter gene expression in muscle as much as 200-fold over the levels found with injection of DNA in saline alone (126–127). There are several mechanisms by which PVP enhances gene delivery. *In vitro* studies demonstrate that PVP stabilizes DNA against degradation by DNAases. *In vivo* immunohistochemical studies demonstrate that PVP also increases both the number of cells expressing a reporter gene and their distribution within the injected muscle (Figure 4-1) (126–127) compared to DNA injected in saline. This suggests that PVP enhances the dispersion and bioavailability of DNA within the injected tissue.

Kinetic studies also demonstrate that formulation of plasmid DNA with PVP extends the range of dose responsiveness. As described above, the level of expression in muscle after injection of pure DNA in saline is saturable as a function of the dose administered. The injection of 50–100μg of DNA in saline into rodent muscles provides maximal levels of gene expression (96, 100); whereas, the dose response in the same models using PVP continued to increase with >100μg of DNA (126) (figure 4-2). The ability of PVP formulations to extend the dose response kinetics suggests either that the plasmid DNA enters cells by a different pathway than "naked" DNA (84, 100, 104) or that the PVP alters the capacity of a rate-limiting step on this pathway. This suggests that PVP not only enhances the stability and dispersion of DNA, but actively enhances the uptake of DNA by muscle cells probably via hydrophobic interaction with the cell membrane.

Studies demonstrate that there is a thermogenic interaction between PVP and DNA that may involve hydrogen bonding or hydrophobic interaction (126–127). Other polymers, which have similar osmotic and colloidal properties to PVP but do not interact with DNA, fail to increase the effectiveness of gene delivery over saline alone (126). These data suggest that formation of a complex between the DNA and PVP is related to its ability to enhance gene delivery.

Condensed Systems: Cationic Lipids

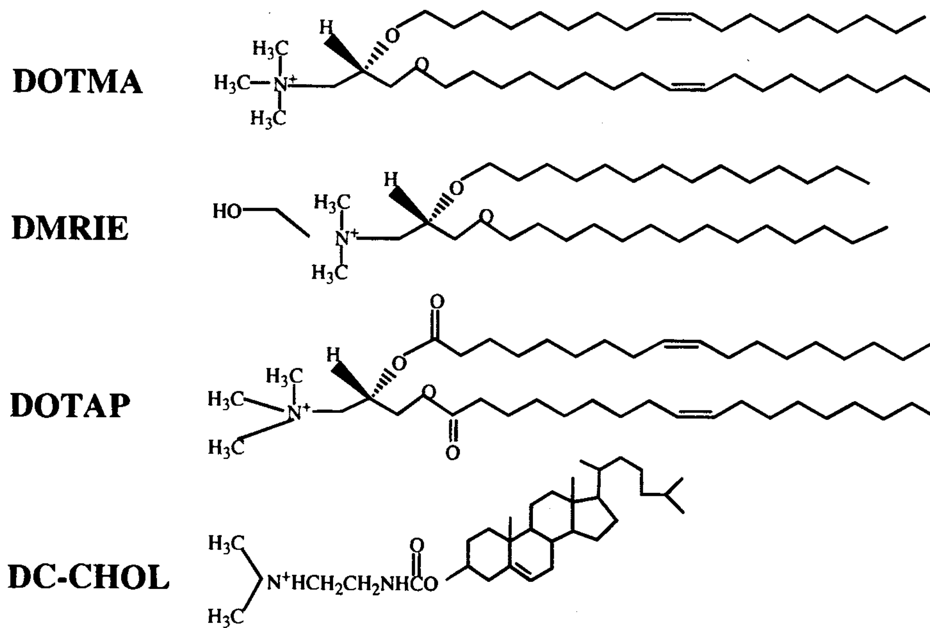
Cationic lipids are important reagents for gene transfer *in vitro* where they enhance the uptake of genetic material into many different types of cultured cells. *In vivo*, cationic lipids may enhance gene delivery in several ways including: (i) protecting DNA against degradation, (ii) modifying the size, charge, and surface characteristics of the DNA-containing particulate to control its biodistribution within the body and access to the target cell, (iii) enhancing the interaction of DNA with the surface of the target cell, (iv) inducing endocytosis, (v) enhancing release of DNA from the endosome and (vi) enhancing the entry of DNA into the nucleus.

Formulations with cationic lipids are the most effective of several lipid-based approaches for gene delivery. One of the first approaches for *in vivo* gene delivery employed liposomes composed of lactosylceramide to encapsulate DNA and target genes to the asialoglycoprotein receptor on hepatocytes following intravenous administration (130). While hepatic targeting of DNA was demonstrated, most of this material was located in the Kupffer cells. The low efficiency of gene transfer observed with conventional liposomes led to studies with pH-sensitive liposomes that were designed to be taken into cells by endocytosis and fuse with the lipid membranes in the acidic environment of the endosomes, thus releasing the encapsulated gene from the endosome into the body of the cell (131–132). Comparative *in vitro* studies demonstrate that liposomes of dioleoylphosphatidylcholine (DOPC):cholesterol hemisuccinate morpholine salt (CHEMS) or phosphatidylserine (PS):cholesterol (Chol) are unable to transfect certain cultured cells that can be transfected with pH-sensitive liposomes (dioleoylphosphatidylethanolamine (DOPE):CHEMS) (133). Proteoliposomes containing Sendai virus glycoproteins to mediate cellular entry and endosomal release have also been used for gene transfer *in vitro* and *in vivo* (134–135).

Formulations of DNA with cationic lipids have been shown to be more effective than conventional liposomes for gene delivery *in vitro* and *in vivo*. The prototype cationic lipid for gene transfer is DOTMA (136–138) (Figure 5-1). Gene transfer is achieved using formulations of DNA with a cationic lipid in combination with a neutral phospholipid such as DOPE. The resulting lipid:DNA complex is not a liposome, but rather a condensed nanoparticle formed by ionic interaction between the cationic lipid and negatively charged DNA and subsequent hydrophobic interactions between the lipid moieties. Electron microscopy reveals a lipid:DNA complex having a "fingerprint-like" internal structure consistent with the picture of condensed, lipid-coated DNA (Figure 5-2). The DNA in this formulation is protected from the environment and exhibits increased resistance to DNAase I or ultrasonication *in vitro* (139) as well as degradation by nuclease in serum or tissue homogenates (80).

The size and surface charge of the lipid:DNA complex can be controlled by altering the stoichiometry of lipids and DNA as well as the method for formulation. The mean size of the colloidal lipid:DNA complex increases as a function of concentration of cationic lipid (for instance from 110 nm for a 1:0.5 (−/+) DNA/DOTMA charge ratio to 220 nm for a 1:3 (−/+) ratio) (Figure 5-3) (80). For a fixed lipid:DNA ratio, the increase in DNA concentration also results in an increase in mean particle diameter. The ratio of DNA to cationic lipid also determines the surface charge of the particle (Figure 5-4). For example, the zeta potential changes from a negative (−55 mV) to a positive value (+55 mV) for a 1:0.5 and a 1:3 (−/+) DNA/lipid charge ratio, respectively. Positively charged complexes can be prepared with a monodisperse size and consistent internal structure as observed by electron microscopy. Negatively charged complexes often contain a significant amount of free DNA and a less defined DNA/lipid particulate structure.

In vitro studies demonstrate that effective transfection of most cell types requires particles formulated to have a net positive charge (138, 140). This positive charge is thought to interact with the negative charge of the cell membrane leading



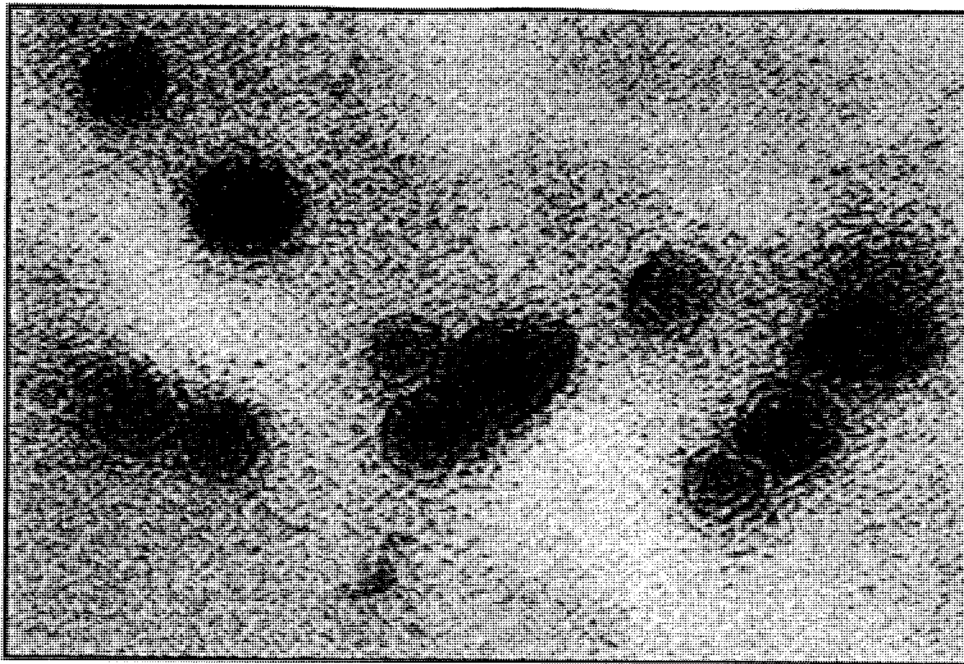


Fig. 5. Cationic lipid delivery systems. (A) Components of cationic lipids currently in clinical trials. DOTMA = 1,2-dioleyloxypropyl-3-trimethyl ammonium bromide; DMRIE = 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DDAB = dimethyldioctadecyl ammonium bromide; DOTAP = 1,2,-dioleoyloxy-3-(trimethylammonio)propane; DC-CHOL = 3 [N-(N',N'-dimethylaminoethane)carbamoyl]-cholesterol; DOPE-dioleoylphosphatidylethanolamine. (B) Transmission electron micrograph of positively charged DNA/DOTMA:DOPE complexes (1:2.5 −/+) showing characteristic fingerprint pattern of a condensed DNA complex. (*Data from ref 80*) (C) Hydrodynamic diameter of lipid:DNA complexes as a function of the charge ratio (+/−; lipid:DNA) and concentration of DNA. (*Data from ref 80*) (D) Zeta potential of DNA/DOTMA:DOPE complexes as a function of charge ratio (+/−; lipid/DNA). (*Data from ref 80*)

to endocytosis (141–142). Particle size also affects transfection efficiency, with particles 300–700 nm in diameter reported to be more effective than small particles 50–100 nm in diameter (138).

Various cationic lipids have been used for gene transfer *in vitro* and *in vivo* including DOTMA and analogous lipids such as DMRIE (138) and DOTAP (143). Gene transfer has also been demonstrated using cationic lipids containing polylysine (144, 145), cholesterol (146, 147), lipopolyamides (148–151), and quaternary ammonium detergents (152). While differences in activity have been described using different cationic lipids with various cell lines, no general structure-activity relationship

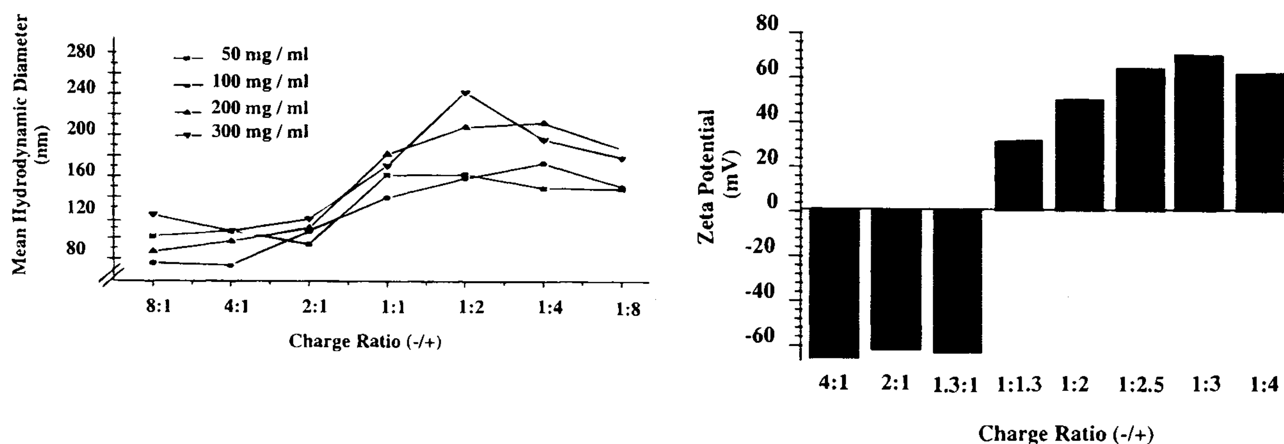


Fig. 5 Continued.

has emerged between the structure of the charge groups or fatty acid component and gene transfer efficiency (138). It is not known, in fact, whether the cationic lipid has any specific role in gene uptake by cells. The function of the cationic lipid may be largely indirect. By condensing the DNA and generating a particle with specific colloidal properties (size, charge surface characteristics), cationic lipids may control the distribution of the DNA-containing particle within the body, cause the particle to interact with the surface of the target cell, and induce endocytosis. Effective gene transfer has been achieved using complexes in which the cationic lipid is replaced by cationic, cyclic peptides such as gramicidin S (153). While cationic lipids with different chemical properties may create particles with different characteristic properties (size, charge, surface, stability), these properties are also dependent upon the stoichiometry of the cationic lipid:colipid:DNA complex and method for formulation. Differences in gene transfer efficiency have also been observed using different counterion salts of the cationic lipid (154).

In contrast to the non-specific role of the cationic lipids, the colipid may play an active role in the internalization of DNA into the cell. Most cationic lipids are ineffective mediators of gene transfer in the absence of a colipid. It has been hypothesized that the addition of the colipid leads to fusion between the lipids in the complex and the endosomal membrane, destabilizing the endosome and releasing the DNA or lipid:DNA complex into the body of the cell (142). Various different colipids have been used for gene transfer including DOPE (136), monooleoglyceride (MOG) (Szoka et al., unpublished data), and cholesterol (95, 156). Optimization of the colipid in the lipid:complex may significantly enhance gene delivery efficiency.

The pathway by which plasmid DNA enters the nucleus after endocytosis and endosomal release has not been extensively characterized. Following exposure of cells to the lipid:DNA complex *in vitro*, DNA can be detected in several intracellular compartments including large perinuclear complexes, vesicles and the cytoplasm. It is not clear at which point in this process the cationic lipid:DNA complex dissociates though such dissociation is essential to achieve expression of the gene within the nucleus (141). It has been suggested that the uptake of DNA from perinuclear or cytoplasmic compartments to the nucleus may be a rate limiting step in gene transfer with cationic lipids (141). It has been demonstrated that addition of a nuclear localization peptide (133) or encapsulation of nuclear proteins (157–158) with the plasmid DNA in proteoliposomes enhances gene transfer efficiency.

Lipopolyamines, such as lipospermine, provide effective *in vitro* gene transfer even in the absence of a colipid (159–160, 148, 151). Lipopolyamines, like other cationic lipids, bind to DNA and form a condensed nanoparticle through both ionic and hydrophobic interactions that enters cells via an ionic interaction with the cell membrane leading to endocytosis (159–160). It has been suggested that lipopolyamine may further enhance endosomal release by buffering the DNA against degradation in the endosome and may facilitate nuclear translocation via the natural tropism of polyamines for the nucleus (159–160). Other lipids can be complexed with the lipopolyamine:DNA complex by hydrophobic interaction to target uptake of these particles. For example, galactosylated lipids with a triantennary galactosyl residue have been used to enhance the uptake of DNA into hepatoma cells expressing the asialoglycoprotein (galactosyl) receptor (160). Significantly, receptor-mediated uptake can be demonstrated using particles without a net positive charge, suggesting that the interaction with the target cell is mediated by the asialoglycoprotein receptor rather than ionic interactions with the cell surface.

Cationic lipid formulations have been used to deliver genes to several targets *in vivo*. Various cationic lipids have been used to enhance gene delivery to epithelial cells of the lung after airway administration (66–68, 161–165). Different formulations have been used to target DNA to the endothelium of the lung (66, 161, 166–167), blood cells (168–169), or tumors (170) after intravenous administration. Cationic lipids have also been used to enhance gene uptake by endothelial cells after direct application to endothelial surfaces (107, 171–176), solid tumors after interstitial administration (177) and ovarian cancer cells after intraperitoneal administration (178).

The most intensively studied target for lipid:DNA complexes is the epithelial surface of the airways in the lung. Immunohistochemical and histochemical studies of lung tissue after intratracheal administration of cationic lipid:DNA complexes have shown expression of gene products to be present within the epithelial cell layers lining the bronchus (67, 161, 167). Cationic lipids can mediate high efficiency gene delivery to these cells, with >50% of cells staining positive for immuno-

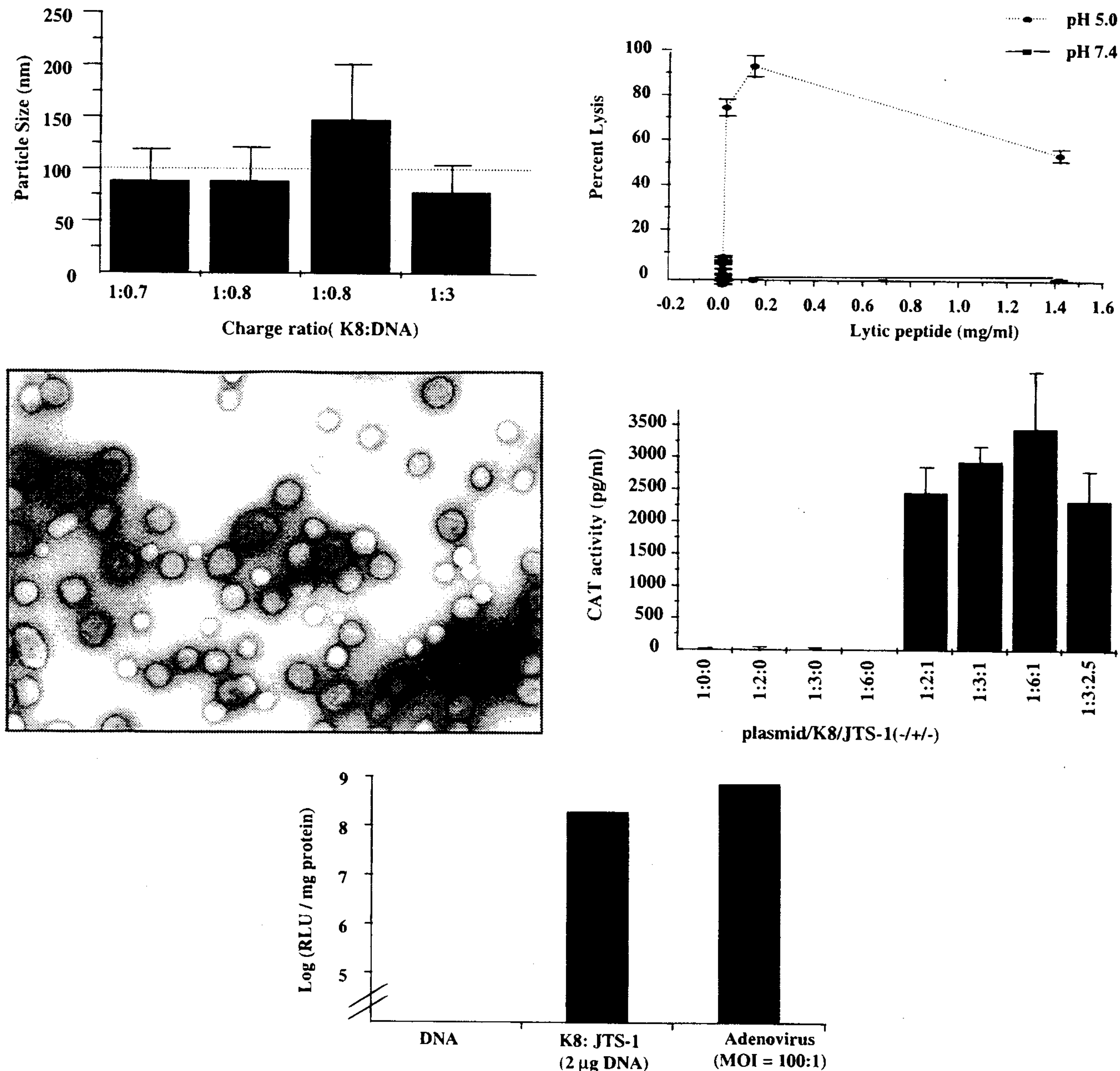


Fig. 6. Peptide-based delivery systems. (A) Mean hydrodynamic diameter and stability of DNA/condensing peptide complexes at various charge ratios. (*Rolland et al., unpublished data*) (B) Hemolytic activity of JTS-1 and INF-7. To serial diluted peptides erythrocytes were added at pH 7.0 (open) and 5.0 (filled). After sixty minutes the hemolytic activity was determined. (*Data from ref 179*) (C) Transmission electron micrograph of K8:DNA complexes. (*Rolland et al., unpublished data*) (D) Comparison of gene transfer (b-galactosidase) into HepG2 cells using (A) 'naked DNA', (B) a peptide-based gene delivery system composed of the condensing peptide K8 and the endosomolytic peptide JTS-1, (C) an adenoviral vector at an MOI of 1:100 (*Data from ref 194*).

reactive protein (161, 163, Petrak *et al.*, unpublished data). The expression, however, tends to be patchy, suggesting that the bioavailability of the complex to the surface is not uniform. The distribution of DNA within the bronchial tree can be varied by altering the characteristics of the formulation and method of formulation. After instillation, gene expression is apparent primarily in the large and mid size bronchioli (66, 142). Many pulmonary diseases such as cystic fibrosis, emphysema or asthma are localized in the lower airways. Controlled nebulization or aerosolization of the formulated material to specific sizes will be required to deliver genes to the alveolus or small and mid-size bronchi.

DNA can also be targeted to the endothelium of the lung after intravenous administration (66). The mechanism of this targeting is not completely understood. It has been demonstrated that complexes with a net positive charge are preferentially targeted to the lung, while particles with a net negative charge comprised of the same components in different ratios are preferentially targeted to the liver (reticuloendothelial cells) (Szoka *et al.*, unpublished data). This suggests that targeting involves

an ionic interaction between the lipid:DNA complex and the endothelial surface of blood elements (Szoka *et al.*, unpublished data). Several mechanisms have been proposed for lipid-mediated targeting of complexes to the lung after intravenous administration. Particulates with a diameter >5–7 μ are known to lodge in the pulmonary capillary beds. While aggregation of the 100–200 nm cationic lipid:DNA complexes in the blood could lead to passive targeting by this mechanism, no evidence for embolization in the lung has been observed histologically. A more likely mechanism is that there is a "first pass" clearance of cationic lipid:DNA complexes by the endothelium in the lung, which is the first capillary bed to be encountered after intravenous administration. This clearance may be due to ionic or hydrophobic interactions between the particle, blood elements, or the capillary endothelium.

Cationic lipid formulations have also been shown to target organs other than the lung, liver and reticuloendothelial cells including cells of the bone marrow (140, 169). While these empirical observations demonstrate that lipid:DNA complexes can be targeted to different tissues, there is an incomplete understanding of the mechanisms by which this targeting occurs. Interpretation of current data is complicated by the fact that cationic lipid:DNA complexes can aggregate in the presence of serum and can interact with proteins or cells, thus altering their colloidal properties.

Since the physico-chemical characteristics of the formulated DNA particle are thought to be critical for biological activity, methods for ensuring colloidal stability of the formulation are necessary for clinical applications. Studies have demonstrated that lipid:DNA complexes will aggregate and become turbid over time in the presence of salt or serum proteins (179). Because of this colloidal instability, initial clinical trials using cationic lipids have been performed with a two-vial system that requires the DNA and lipid to be combined shortly before each administration. This has limited the ability to standardize, characterize, and quality control the formulation. Recently, methods have been developed for lyophilizing the formulated material in the presence of specific cryoprotectants to increase the stability of lipid:DNA complexes. Following freeze-drying under controlled conditions with specific cryoprotectants, and rehydration to isotonicity, the characteristics of the complexes (size, zeta potential, complexation efficiency) and transfection efficiency remained unchanged (Bruno *et al.*, unpublished data).

Condensed Systems: Cationic Polymers

Several different classes of cationic polymers have been described to enhance the uptake of DNA into cells and its egress from the endosome. The first class, dendrimers, are polyamidoamine cascade polymers whose surface has a uniform positive charge and whose diameter is determined by the number of synthetic steps. Dendrimer:DNA complexes have been constructed employing dendrimers of different sizes as well as different charge ratios (cationic dendrimer to anionic DNA). These complexes exhibit efficient gene delivery into a variety of cell types *in vitro* (180). The second class, polyethylenimine (PI) (181) or chitosan (Mumper et al., unpublished data), similarly has a high uniform positive charge density, will complex with DNA, and will transfect a variety of cells *in vitro*. Both polymers are capable of condensing plasmid DNA to form particulate complexes with varying size and charge that may interact with the membranes of cells by ionic interaction and enter cells by endocytosis. The polymers may additionally enhance the intracellular trafficing of DNA by buffering the lysosomal compartment to protect DNA against degradation (181).

Polymers may also be used as a scaffold for codelivery of compounds to enhance gene targeting or uptake further. For example, the attachment of peptides with pH sensitive endosomal-lytic activity has been shown to increase the effectiveness of dendrimer-mediated gene delivery ~3 fold (182).

Condensed Systems: Proteins

Formulations of DNA with protein ligands have been developed to achieve receptor-mediated uptake of plasmids into certain target cells. Proteins are commonly complexed with DNA by covalently coupling the protein to polylysine and binding this complex to DNA through an ionic interaction between the positively charged polylysine and the negatively charged DNA (183–194). The interaction of polylysine with DNA also condenses the DNA, and toroidal protein:DNA structures as small as 80 nm diameter have been described (184). The protein component of the resulting complex retains its ability to interact specifically with cognate receptors on the target cell, which leads to internalization of the DNA into the cell by receptor-mediated endocytosis. Several different protein ligands have been used effectively *in vitro*. Transferrin:polylysine:DNA complexes have shown effective gene delivery into various cell types *in vitro* (183) including hematopoietic cells (189), T cells (195), and pulmonary epithelium (190). Asialoorosomucoid:polylysine:DNA complexes demonstrate specific gene delivery and expression in hepatocyte and hepatoma cells (185, 188). Surfactant B:polylysine:DNA complexes (193) and anti-thrombomodulin:polylysine:DNA complexes (191) have been used for effective gene delivery into epithelial airway cells.

Gene delivery has also been achieved by complexing polylysine with small molecule ligands such as folate (196), galactosyl residues (197–198), lactose (198–199), or N-acetylgalactosamine (200). The mechanism of folate-mediated uptake of DNA may differ from that of peptide ligands in that folate is normally taken up through the potosome rather than the endosome (196).

The limiting step in receptor-mediated gene transfer *in vitro* is the rapid degradation of DNA within the endosome after endocytosis (201). Several methods have been described for enhancing the release of DNA from the endosome before fusion with the lysosome acidifies the endosomal compartment and introduces nuclease capable of rapidly digesting the DNA. One approach has been to add non-infectious adenoviral particles (201–203) to the transfection mixture. Adenoviral particles, like many other viral particles, induce endosomal lysis during the process of adenoviral infection (204). Endosomal lysis by adenoviral particles is mediated by the penton protein on the surface of the virus that undergoes a change in tertiary structure upon acidification of the endosome (205). This change in conformation creates a structure that is capable of penetrating and disrupting the endosomal membrane and causing release of the endosomal contents into the body of the cell. When DNA is co-internalized in the endosome with the adenoviral particle, adenovirus-induced endosomal lysis also releases DNA from the endosome into the body of the cell. *In vitro* gene transfer

efficiencies may be enhanced 100–1000 fold by the addition of adenovirus to tissue culture media during receptor-mediated transfection with transferrin:polylysine:DNA (201–203, 206–207, 190), asialoglycoprotein:polylysine:DNA (208–210) or folate:polylysine:DNA (196). The adenovirus most commonly used is a replication defective variant of human adenovirus type 5. Studies have also demonstrated that chicken adenovirus (211) or rhinovirus particles (212) exhibit similar properties. A related method for gene transfer involves coupling polylysine directly to the adenovirus and complexing DNA to the surface of the virus by ionic interactions (209, 213). Internalization of the adenovirus:DNA complex is thought to be mediated by binding of the adenoviral knob proteins to integrins on the cell surface leading to endocytosis and endosomal release.

Despite considerable success with receptor-mediated gene transfer using protein and endosomal release agents *in vitro*, these methods have been generally ineffective *in vivo*. Asialoglycoproteins have been used to target genes to hepatocytes *in vivo* and establish hepatic expression of reporter genes in normal animals (214–216), LDL-receptor in LDL-deficient rabbits (217), albumin in analbuminemic rats (215), and methylmalonyl CoA mutase in mice (78). Gene targeting to hepatocytes has been confirmed using hepatocyte-specific promoters to express recombinant genes *in vivo* (186, 217–218) and histological analysis (216). These studies also demonstrate that DNA is taken into cells through the endosomal compartment.

In vivo studies have revealed several different pathways for intracellular trafficking of DNA following receptor-mediated endocytosis. When plasmid DNA is delivered to hepatocytes in normal animals, DNA is cleared from the liver within several hours leading to gene expression only for several days (78, 214–215, 217–218). In contrast, when the same material is injected into animals after a partial (subtotal) hepatectomy (216, 218) or treatment of animals with colchicine (219), DNA persists in the liver for several months leading to a prolonged period of gene expression. Studies by Chowdhury *et al.*, (216) demonstrate that partial hepatectomy leads to compartmentalization of intact plasmid DNA within intracellular, membrane encapsulated vesicles. It was hypothesized that this persistence could be related to disruption of microtubules and the progression of the endosome along these microtubules to the lysosome. This hypothesis was confirmed with *in vivo* studies demonstrating that the administration of colchicine to animals before treatment with asialoorosomucoid:polylysine:DNA complexes leads to persistence of DNA in the liver and gene expression for 8–10 weeks (219).

The major factors limiting the *in vivo* effectiveness of protein:DNA complexes may be their poor bioavailability to many target cells and their colloidal instability in physiological fluids. Bioavailability may be limited by the size of the complex (particularly complexes incorporating adenoviral particles) as well as by aggregation and/or dissociation of these complexes in physiological fluids. Moreover, it has been difficult to achieve reproducible effects due to the intrinsic variability in the quality and size of polylysine, the covalent protein:polylysine complexes and methods used for producing formulations.

Condensed Systems: Peptides

Peptide systems are designed to condense and stabilize DNA to improve its bioavailability to selected target cells, induce the uptake of DNA into cells through a receptor-mediated process, and enhance the intracellular trafficking of DNA from the endosome to the nucleus. There are three functional elements in a peptide-based formulation. The first is a condensing function mediated by a cationic peptide. The second is receptor-binding function mediated by a peptide or glycopeptide ligand. This targeting ligand can be combined with the cationic peptide as a single chain through a labile spacer. The third is an endosomal release function mediated by a peptide designed to mimic the endosomal release function of the adenoviral penton protein. A critical feature of peptide formulations has been the development of peptides of minimal length to reduce the risk of immunogenicity (194).

To establish the minimum size of a cationic condensing peptide, a series of peptides with the sequence YKAK WK with n = 4, 5, 6, 7, 8, 10 and 12 lysines in the central cationic cluster were synthesized and tested for their ability to condense DNA and enhance gene transfer in cultured cells. Peptides as small as YKAK WK (K8) effectively condensed DNA and formed a positively charged complex. Incubation of hepG2 cells with this peptide:DNA complex resulted in a 50–100 fold increase in reporter gene expression compared to the incubation of hepG2 cells with DNA alone (194). The efficiency of transfection with these condensed particles alone is poor compared to transfection with cationic lipids or recombinant viruses. This poor efficiency of transfection is though to be due to destruction of the complex in the endosome after endocytosis as observed for protein:DNA formulations (206, 220–221, 209–210, 201–203).

Endosomal release can be mediated by fusogenic peptides such as the influenza virus hemagglutinin peptide. Unlike the penton protein of adenovirus, whose functional determinants have not been specifically localized, the minimal sequence of the influenza hemagglutinin has been mapped and its structure-activity relationships characterized in detail. The active influenza hemagglutinin peptide has a globular structure at neutral pH and assumes an amphipathic helical structure at acid pH that is capable of penetrating and disrupting endosomal membranes. Synthetic peptides have been developed for enhancing protein delivery to cells which share this property (222–223). The influenza hemagglutinin peptide has been covalently coupled to polylysine and used in conjunction with protein formulations to enhance gene transfer. While this covalently modified peptide enhances the efficiency of gene transfer, the effect was significantly less than with adenovirus (197, 206, 221–222).

Molecular modelling has been used to design novel amphipathic peptides to enhance endosomal release. A peptide of 20 amino acids (GLFEALLELLESLWELLLEA, JTS-1) has been shown to be more potent than influenza virus hemagglutinin in erythrocyte lysis assays (194). Complexes of this fusogenic peptide JTS-1 with the condensing peptide with K8 have been produced by the addition of JTS-1 to the positively charged K8:DNA complex. The resulting K8:JTS-1:DNA complex provides high levels of gene expression in a variety of cell lines. The efficiency of gene transfer with K8:JTS-1:DNA was compared to recombinant adenovirus containing the same expression cassette as the plasmid (194). Maximal luciferase activity using a 100:1 MOI (pfu:cell) of adenoviral particles was about 1×10^9 light units per mg protein, ~10-fold higher than the maximal level of gene expression achieved using K8:JTS-1:DNA complexes. The maximal level of gene expression

achieved with peptide formulations was equivalent to a 30:1 MOI (pfu:cell) of adenovirus. Significantly, studies in mice have demonstrated that the peptide formulations are non-immunogenic (194).

The observed difference in the maximal (saturating) level of expression achieved using recombinant adenovirus and K8:JTS-1:DNA suggests that the rate limiting step in the delivery, uptake or expression of the plasmid differs from that of adenovirus. One potential site for this rate limiting reaction in plasmid activity is nuclear uptake (194). After entry of a wild-type adenovirus particle into the cytoplasm during infection, the adenoviral capsids remain intact and are translocated to the nuclear pore for efficient viral DNA delivery into the nucleus (223). This process is thought to be mediated by nuclear targeting sequences on the adenoviral capsid protein. So too, adenoviral vectors may specifically enhance nuclear uptake of therapeutic genes thus enhancing a potentially rate limiting step. Another potential limiting step in plasmid delivery is the interaction of the particle with the surface of the target cell (194). The peptide formulations reported to date do not incorporate targeting ligands and appear to interact with cells by a non-specific ionic interaction. The low affinity and efficiency of this interaction may limit the efficiency of gene transfer. For example, for receptor-dependent gene delivery using protein formulations, the transduction efficiency of cells correlates with expression levels of the specific receptor (209–210, 194, 221, 224).

CONCLUSIONS

Gene therapy has enormous promise as a strategy for providing safe and effective therapies for many common disorders. To fulfill this promise, approaches to gene therapy must be developed that are consistently effective and are also as safe and cost-effective as conventional pharmaceutical and biotechnology products. These are familiar clinical challenges in drug development challenges against which methods for gene therapy and progress towards developing gene therapy products must be measured.

This review has described two parallel themes in the ongoing development of gene therapy. The first is the preclinical and clinical evaluation of prototype approaches to gene therapy approaches involving the administration of genetically engineered cells and viruses as well as plasmid DNA. These approaches demonstrate significant pharmacological effects in many animal models of disease and may lead to the first approved gene therapy products within a decade. When measured against the standards of conventional pharmaceutical products, however, the limitations of current methods are apparent. For example, the lack of established transplantation methods for cell-based therapies, the toxicities of virus-based therapies, the limited *in vivo* potency of some plasmid-based approaches limit the applicability of these approaches to gene therapy. Moreover, many proposed therapies do not map well to clinical practice, raise complex regulatory issues, and may not provide economic advantages over current therapies.

The second theme is the emerging pharmacological approach to gene therapy that is based on an advancing understanding of the DNA as a chemical entity, the biological barriers to effective gene therapy, and pharmaceutical experience in formulation and drug delivery. This approach has already led to the development of novel methods for gene delivery that are as effective as viral vectors for gene delivery to certain somatic targets. Perhaps most importantly, this pharmaceutical approach provides a theoretical framework for future advances in this field. A critical challenge is to understand the pathway by which DNA enters the cell and penetrates to the nucleus. More information is needed about the physical structure and thermodynamics of DNA condensed with carious carriers as well as the colloidal and surface properties of these particles. More investigation is needed to understand the fate of DNA complexes in various extracellular and intracellular compartments. It is important to understand how these complexes interact with tissues and with physiological fluids and how these interactions affect gene delivery and gene expression. From such information, it will be possible to identify those cells and diseases that are- or are not-appropriate targets for gene therapy and selectively apply the materials and methods of drug delivery and formulation to engineer effective gene therapy products.

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