The AAPS Journal 2007; 9 (1) Article 2 (http://www.aapsj.org).

Themed Issue: Non-viral Gene Delivery: Opportunities and Challenges
Guest Editors - Dexi Liu and Craig K. Svensson

Vectors for Airway Gene Delivery

Submitted: October 19, 2006; Accepted: December 18, 2006; Published: January 19, 2007

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Abstract

Delivery of genes to the airway epithelium for therapeutic purposes seemed easy at first, because the epithelial cells interface with the environment and are therefore accessible. However, problems encountered were more substantial than were originally expected. Nonviral systems may be preferred for long-term gene expression, for they can be dosed repeatedly. Two nonviral gene transfer systems have been in clinical trials, lipid-mediated gene transfer and DNA nanoparticles. Both have sufficient efficiency to be candidates for correction of the cystic fibrosis defect, and both can be dosed repeatedly. However, lipid-mediated gene transfer in the first generation provokes significant inflammatory toxicity, which may be engineered out by adjustments of the lipids, the plasmid CpG content, or both. Both lipid-mediated gene transfer and DNA nanoparticles in the first generation have short duration of expression, but reengineering of the plasmid DNA to contain mostly eukaryotic sequences may address this problem. Considerable advances in the understanding of the cellular uptake and expression of these agents and in their practical utility have occurred in the last few years; these advances are reviewed here.

Keywords: DNA nanoparticles, gene therapy, cystic fibrosis, lung, airway epithelium

Prospect for Gene Therapy: Opportunities and Technical Obstacles

The genetic basis for human disease is now understood for a large number of disorders. From a diagnostic perspective, this information permits a molecular diagnosis for a given individual. From a therapeutic perspective, this information has been important in establishing animal models of human disease, which has greatly assisted in the identification of efficacious candidate drugs. The prospects for directly using DNA as a therapeutic, however, have been problematic for most diseases because of several technical limitations. Initially, viruses were modified to express potential therapeutic genes and most early gene therapy clinical trials employed viral vectors. Viral vectors have been successful in expressing genes in bone marrow following ex vivo gene transfer, with these gene-modified cells then transplanted into autologous subjects, although cancer induction in some subjects underscores the potential toxicities of vectors that integrate into chromosomal DNA.[1,2] For in vivo gene transfer in humans, viral vectors have in some tissues, such as lung, suffered from reduced gene transfer efficiencies compared with other animal species because of discordance of receptors on the apical surface membrane.[3-7] Moreover, viral vectors are immunogenic and repeat applications have often resulted in production of neutralizing antibodies by the host, preventing productive readministration.[8-11] Inflammatory responses to some vectors, such as adenovirus, have also resulted in toxic responses, including mortality.[12-14]

These technical obstacles have focused efforts to develop nonviral technologies to introduce and express complementary DNA (cDNA) and interfering RNA (RNAi) moieties in human cells. Although viruses have evolved specific mechanisms that permit them to introduce nucleic acids in human cells, nonviral vectors must address each of these physiologic barriers de novo (Figure 1). The vector must be sufficiently stable after administration to permit bulk delivery to the tissue of interest, which requires protection from potential bloodstream sheer forces, enzymatic degradation of protein and/or its nucleic acid payload, and preservation of the vector's ability to gain access to cells after exposure to host interstitial components, such as albumin and antibodies. In cases such as lung gene transfer, local pulmonary administration may significantly increase the concentration of vector in the proximity of the target cell. The vector must gain entry to the cell by either physical association (positively charged liposomes) or binding to a cell surface membrane receptor that then brings the vector inside of the cell upon internalization. Although the assumption has been that lipid-mediated gene transfer occurs by fusion of the lipids with the cell membrane, some studies show that inhibitors of endocytosis inhibit lipid-mediated gene transfer.[15] Probably the precise composition of the liposomes, including the net charge and lipid composition, influence cell entry.

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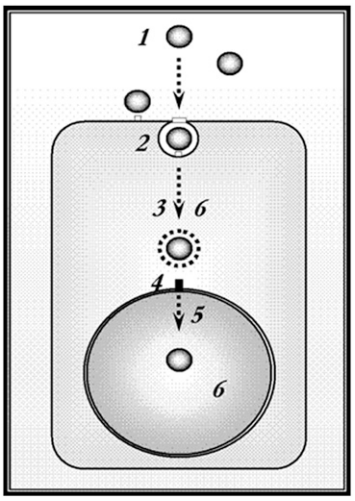

Figure 1. Physiologic barriers for nucleic acid uptake into cells following in vivo gene transfer. These steps include stability after either local administration into target tissues or systemic injection (1), entry into the cell (2), survival in the cytoplasm (3), intracytoplasmic transport to the nucleus (for DNA vectors, 4), nuclear uptake via the 25-27 nm nuclear membrane pore (5), and vector dissolution to release biologically functional nucleic acids (6). If the payload is DNA, this step also requires efficient gene expression, which may optimally require robust and prolonged transgene expression and, in many cases, appropriate posttranslational modification of the encoded protein.

Polyplexes made with polyethylenimine (PEI), on the other hand, appear to enter the cell through more than one mechanism, including clathrin-coated vesicle-mediated endocytosis (which appears to lead to lysosomal degradation) and caveolar entry (which does not lead to degradation and may explain the apparent efficiency of these polyplexes).[16] However, the surface molecules to which either the lipid-DNA complexes or the PEI-DNA complexes bind have not been reported. In some instances, addition of ligands such as transferrin or lactose to the complex has been reported to improve uptake, presumably by targeting the transferrin receptor and surface lectins, respectively.[17-19] Other ligands such as the HIV-1 Tat protein have also been suggested,[20] although with more complex ligands, immunogenicity may become a serious problem. For DNA nanoparticles composed of polylysine–polyethylene glycol (PEG) and DNA, colocalization of the nanoparticles and the protein nucleolin was observed. Moreover, only cells that expressed nucleolin on the cell surface took up the DNA nanoparticles. Although nucleolin is well known to shuttle between nucleus and cytoplasm, in some cells (including airway epithelial cells) it reaches the plasma membrane as well. Purified nucleolin binds to DNA nanoparticles with KDs in the low nanomolar range, by surface plasmon resonance, and maneuvers that deplete surface nucleolin also reduce expression of reporter genes from DNA nanoparticles. Moreover, transfection of tagged nucleolin that migrates to the surface increases gene expression from DNA nanoparticles. Preincubation of the particles with exogenous nucleolin results in dose-dependent reduction in gene expression. Thus, nucleolin is an excellent candidate for the receptor for the DNA nanoparticles.[21]

A high-affinity association between vector and cell surface receptor is necessary but not sufficient for successful in vivo gene therapy. The intracellular trafficking pathway of most surface receptors is degradative in character, with endosomal uptake of the receptor and payload leading to intracytoplasmic fusion with lysosomes, with subsequent enzymatic degradation as well as potential acid denaturation of the vector. Although viruses have evolved specific mechanisms to destabilize lysosomes,[22,23] these moieties, such as influenza virus hemagglutinin HA-2 subunit, are highly immunogenic and therefore not appropriate for use in nonviral vectors developed for chronic human diseases. The chemical structure of PEI has been shown to destabilize lysosomes in vitro because of its protein sponge effect,[24,25] although whether this mechanism is applicable following in vivo gene transfer is less clear in view of likely dilutional effects as well as potential toxicities of the polymer that restrict local concentrations. For DNA nanoparticles, the nucleolin shuttle pathway to the nucleus is nondegradative, bypassing lysosomes, and endosomal release mechanisms are not required. Even if the nonviral vector escapes the lysosomal compartment or enters the cells via a nondegradative pathway, the journey to the cell nucleus is problematic, since free DNA molecules > 2000 base pair in length are essentially immobile in the cytoplasm.[26] This observation may explain why microinjection studies have shown that lipid-DNA complexes injected into the cytoplasm do not access the nucleus for gene expression. Carrier-mediated transport to the nucleus is likely required for typical plasmid DNA payloads,[27] although small DNA oligonucleotides may be sufficiently diffusive to travel to the nucleus after cell entry.[28] The site of action of short interfering RNA (siRNA) is the cell cytoplasm, although short-hairpin RNA expression plasmids must enter the nucleus to be active.

Once it reaches the nucleus, the vector must gain entry. In tissue culture conditions, nuclear membrane disintegration during mitosis appears to be the primary mechanism allowing for entry of DNA vectors into the nucleus.[29-34] In contrast, most cells in the body are postmitotic, and those cells that replicate, such as bone marrow progenitors and epithelial stem cells, have a modest number of cells undergoing cell division during the time course that a nonviral vector would be present in sufficient concentrations to be effective. Unless the vector continues to have a membrane-penetrating capability after entry into the cytoplasm, the only known mechanism for nuclear entry is transit through the nuclear membrane pore, which has a functional diameter of 25 to 27 nm.[35,36] This size limitation restricts entry to essentially

all nonviral vectors that are not formulated as nanoparticle compositions having a minimal diameter less than the nuclear pore diameter.[37] Both PEI and polylysine DNA complexes are successful in accessing the nucleus when they are microinjected into the cytoplasm, and when they are injected into the nucleus, the polycations do not interfere with transcription. Once in the nucleus, the vector, which until this time must have been sufficiently stable in structure to address the preceding physiological barriers, must now become unstable to release the nucleic acid payload to be functional. Specific mechanisms are needed to account for vector dissolution in the nucleus without induction of damage to surrounding chromatin and other nuclear structures. For DNA, the encoded protein then must be expressed at appropriate levels and for an appropriate duration to be therapeutic. Repeat administration of the vector will be required for most chronic disorders, since the prospect for in vivo stem cell gene transfer is not well defined, such transfer is likely to be inefficient, and the mechanism to ensure efficient transfer of the nucleic acid to daughter cells would require a replication competency that would entail another layer of complexity, inefficiency, and potential toxicity.

Although intrapulmonary administration facilitates direct contact of the vector to the target tissue, the lung poses various biological and structural barriers that may limit gene transfer. Lung epithelial cells are covered with a glycocalyx layer composed of complex mucopolysaccharides and glycoproteins, which may impede access of the vector to the apical surface membrane. Compared with other tissue sites, the airway apical membrane surface is relatively devoid of cell surface receptors, thereby limiting options for receptor-mediated uptake of the vector. In various disease states, such as cystic fibrosis, asthma, and bronchitis, mucus secretions and bronchoconstriction may limit access to airway epithelium and restrict airflow to these cells. These considerations suggest that effective gene transfer in patients with active airway inflammation may be difficult and that optimizing the clinical status of the subject may be important in the design of clinical trials.

Nanoparticle Nonviral Vectors Couple Efficacy and Safety

Initial efforts to condense DNA with polycations, such as polylysine and PEI, generated complexes that consisted of many molecules of DNA and that had diameters greater than 50 nm—in most cases, greater than 100 nm.[38-40] The effectiveness of these compositions in vivo was modest, with improved hepatic expression following partial hepatectomy, and for many, cell injury and gene transfer may have been linked. Hanson and Perales were successful in generating condensates consisting of single molecules of DNA and a particle size <25 nm.[41-43] These complexes were formulated by adding polylysine to DNA at a salt concentration sufficiently high to permit positive cooperativity in lysine binding to DNA, which facilitates unimolecular DNA condensation. Although effective in mice and capable of targeting specific tissues, such as lung tissues,[44] compositions containing typical plasmid vectors between 3 and 10 kbp were stable in only hypertonic saline, typically at concentrations approaching or exceeding 1M NaCl.

To prepare small nucleic acid complexes that are stable in saline, other investigators have modified the formulation procedure and components to prepare highly stable nanoparticles. DNA was compacted as unimolecular nanoparticles using lysine peptides of defined composition, and stability in saline was achieved by covalently modifying the lysine peptide with PEG.[37,45] For the lung, a preferred condensing peptide consists of a 30-mer lysine peptide with an N-terminal cysteine, to which 10 kDa PEG is coupled (CK_{30}PEG10k).[45] As developed by Copernicus Therapeutics, these DNA nanoparticles have a homogeneous size and volume distribution, are stable in saline at concentrations of at least 12 mg/mL of DNA, and are stable in saline for >3 years at 4°C, 9 months at room temperature, and 1 month at 37°C.[37,46] As visualized by electron microscopy (Figure 2), these nanoparticles have distinct shape parameters based on the lysine amine counterion present at the time of DNA mixing.[37,45-48] The nanoparticles are spheroids if trifluoroacetate (TFA) is the counterion, whereas rodlike forms are observed if acetate is the counterion. Other counterions, including chloride, bromide, and bicarbonate, also give characteristic shape distributions, including toroids. In each instance, the calculated volume distribution demonstrates unimolecular DNA compaction, as Liu et al found for TFA nanoparticles (Figure 2C).[37]

If sufficiently small, DNA nanoparticles robustly transfect nondividing cells.[37] This phenomenon was first studied by comparing the ability of naked or compacted DNA encoding enhanced green fluorescent protein (EGFP) to transfect cells following intracytoplasmic or nuclear injection. Cells receiving intracytoplasmic injections of DNA were monitored for cell division by coinjecting high-molecular-weight rhodamine-labeled dextran, which is unable to cross the nuclear membrane unless the cell divides. Enhanced gene transfer in nondividing cells was observed for compacted DNA compared with naked DNA following an intracytoplasmic injection, but only for nanoparticles having a minimum diameter less than 25 nm, which approximates the size of the nuclear membrane pore. All gene transfer was blocked by wheat germ agglutinin, which is known to block the nuclear membrane pore. These studies indicate that appropriately small DNA nanoparticles can productively transfect nondividing cells, gaining entry into the nucleus by crossing the nuclear membrane pore. This microinjection study

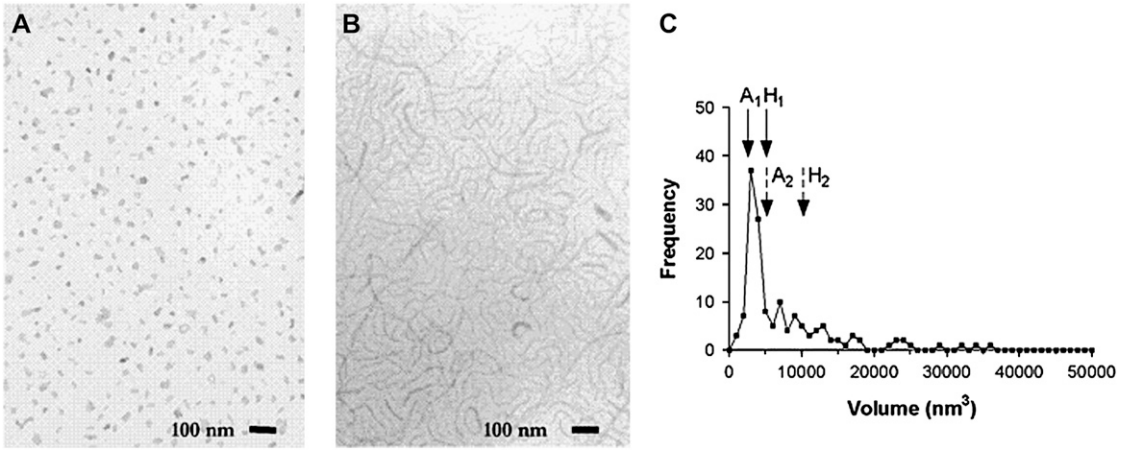

Figure 2. Electron micrographs of the same 5.4-kbp plasmid compacted with CK_{30}PEG10k polylysine peptide having either TFA (A) or acetate (B) as the amine counterion at the time of DNA mixing. Nanoparticles were subsequently concentrated in saline to 4 mg/mL of DNA. (C) A volume histogram of a 2.9 kbp plasmid compacted with CK_{30}PEG10k having TFA as counterion. Maximum and minimum diameters were determined using the Sigma Scan program, and volumes were calculated based on ellipsoidal shape. A_1 and H_1 refer to the calculated anhydrous and hydrated volumes, respectively, of a single molecule of DNA and charge equivalent amount of lysine, as previously described.[37] A_2 and H_2 note the calculated volume of a complex containing 2 molecules of DNA. Reprinted with permission from Liu et al.[37] PEG indicates polyethylene glycol; TFA, trifluoroacetate.

correlates well with in vivo transfection results with a series of postmitotic target cells, including lung epithelium (30%-80% transfection efficiency),[45] retina (99%),[49] and brain (brisk gene transfer around needle track).[50] DNA nanoparticles gain entry into these cell types by binding to cell surface nucleolin,[51] which facilitates nuclear transport and uptake in a nondegradative pathway. These results provide a mechanistic understanding of how these DNA nanoparticles are able to address the various physiologic barriers summarized in Figure 1.

Despite the handicap of poor nuclear access, lipid-mediated gene transfer and expression occurs in the airway, and it is likely that the specific lipid composition plus the large amount of DNA delivered to the cytoplasm affect the results. Lipid-mediated gene transfer into airway epithelium has been demonstrated in animals and humans.[52-56] However, for the current generation of lipid-mediated gene transfer, significant and limiting inflammatory toxicity was encountered in humans at the doses required to achieve even modest correction of the cystic fibrosis (CF) defect. This toxicity appears to be the consequence of CpG sequences in the plasmid, which interact with toll like receptor 9 (TLR9) receptors to stimulate production of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor (TNF)α, and IL-12. Depletion of CpG sequences in the plasmid greatly reduces the inflammatory toxicity and improves the therapeutic index.[57,58] These maneuvers also prolong the apparent half-life of expression from the plasmid. In the liver, it is desirable to eliminate all bacterial sequences in the plasmid in order to prolong expression, including the bacterial origin of replication, antibiotic resistance sequences, and other bacterial DNA.[59,60] The promoter and other remaining elements should be of eukaryotic origin. These "minicircles" have protracted high-level expression in the liver, with expression persisting for years[61]; the efficacy of minicircles in the lung has not been tested. The extent to which modification of the plasmid to remove inflammatory sequences will increase expression and prolong half-life of the transferred gene has not yet been reported.

DNA nanoparticles, even those that retain CpG sequences, appear not to display the consequences of TLR9 activation.[62,63] This may be due to one of several mechanisms. First, it is likely that the nanoparticle remains compacted as it enters the nucleus, and so free DNA is never available to the cytoplasmic TLR9. In addition, it is likely that the DNA nanoparticles enter only cells with surface nucleolin. If these do not include the professional immune cells with the greatest density of TLR9, it may be that much of the lung response is avoided. Avoiding the CpG response gives greater flexibility in plasmid design, since at least one excellent eukaryotic promoter, ubiquitin C, has CpG sequences that cannot be eliminated without also eliminating promoter function. There also appears to be little excess inflammation in the lungs of mice over that induced by saline alone by DNA nanoparticles. Mice treated with 100 μg of DNA in PEG-polylsine (polyK)-DNA nanoparticles displayed some modest accumulation of mononuclear cells about the pulmonary veins.[63] One possible mechanism for such a response is that if the DNA nanoparticles separate into their component parts, the polycation might be available to activate complement,[62,63] but no complement consumption was detected in either animal models or human studies, so the mechanism of the modest mononuclear cell accumulation remains unclear. However, there was no systemic inflammatory response.

In contrast, significant inflammation has been reported for PEI-based gene transfer complexes applied to the lung, although the exact origins of this response, and therefore, whether it can be engineered out by plasmid modification, are unclear.

Intrapulmonary administration of nonviral vectors will require formulations that are effective after aerosol generation. Compositions must be stable to the shear forces found during mist production, and other variables, such as solution viscosity and surface tension parameters, affect droplet size. For CF, proximal airway delivery is desired, and for humans a droplet size of 3 to 5 μm is optimal. Smaller droplet sizes (1-2 μm), in concert with large tidal volumes and breath holds, facilitate vector delivery to the alveoli. DNA nanoparticles can be aerosolized using multiple types of aerosol generators and remain structurally intact and biologically active in intubated animals. Consistent transgene expression has been observed throughout the lung, and modification of aerosol droplet sizes and airway dynamics have been used to achieve either proximal or deep lung delivery and transgene expression.

Human CF Clinical Trials

Both DNA nanoparticles and lipid-mediated gene transfer have moved to clinical trials for patients with CF. Lipoplexes made with cystic fibrosis transmembrane conductance regulator (CFTR) cDNA and lipid 67 were used in clinical trials in the nose and the lung in CF patients in Great Britain.[55] No messenger RNA (mRNA) for CFTR was detected in epithelial samples. Modest improvement in chloride transport, which was not dose related, was detected at both sites by transepithelial potential difference measurements, but at a cost of significant and limiting systemic inflammatory response. In other trials in the United States, little or no chloride transport improvement could be documented with lipid-DNA complexes, but significant inflammatory responses were observed in these trials.[52,56] The inflammatory toxicities were consistent with CpG responses. The newer CpG-depleted plasmids have not been tested clinically but appear promising in animal studies. DNA nanoparticles administered to the nasal epithelium of CF patients also gave no detectable vector-specific mRNA in nasal scrapes, although the DNA vector persisted within epithelial cells for at least 2 weeks in most samples.[62] DNA nanoparticles produced no inflammatory response or other detectable toxicity and produced partial correction of the CF chloride transport defect. Four of 12 patients reached chloride transport values in the normal range at least once in the course of the study. For both lipid 67-DNA complexes and DNA nanoparticles, duration of detectable correction was brief—for most patients, less than 2 weeks. DNA nanoparticles can be aerosolized and in animal models are well distributed throughout the lung; they transfect as well after aerosolization as before. They are moving toward clinical trials in the lung.

Summary and Conclusions

To deliver genes to the airway epithelium for therapy of CF and possibly other hereditary diseases, such as alpha 1 antitrypsin deficiency, or acquired conditions such as asthma, vectors must be capable of aerosolization, nontoxic, capable of repeat dosing, and sufficiently efficient to allow the therapeutic gene to effect biological change. The requirement for repeat dosing has focused attention on nonviral vectors, and the requirement to minimize toxicity for lipid/DNA complexes has driven investigators to reengineer plasmid DNA to reduce inflammatory sequences. Moreover, elimination of bacterial sequences may improve the duration of transgene expression. Besides these criteria, it is critical that the vectors be able to be constituted reproducibly and to be sufficiently chemically defined as to be of pharmaceutical quality. Two vector types appear to meet these criteria: DNA nanoparticles composed of both PEG-polyK-DNA and lipid-DNA complexes have come to clinical trial. Both have achieved some success and are under intensive development now for lung clinical trials. Modification of the plasmid DNA is achieving less toxicity and longer duration of transgene expression, and the vectors themselves appear to be sufficient for partial and possibly therapeutic correction of the CF defect. Nevertheless, improvements in vector design may permit lower doses to be administered, thereby reducing dose-related toxicities. For DNA nanoparticles, addition of targeting ligands to the complexes may improve the specificity of gene transfer to airway epithelium, may permit lower doses to be effective, and may address structural lung barriers. For example, complexes incorporating an antibody fragment to the polymeric immunoglobulin receptor access airway epithelium after an intravenous injection,[44] which might permit lung gene transfer in the setting of copious airway mucus and bronchoconstriction. Ligands with cell membrane penetrating activity, such as C105Y,[64] also may be effective after intrapulmonary dosing. Because the modular design of these vectors facilitates assessment of such modifications, it is reasonable to expect further improvements in the composition of nonviral vectors for the lung.

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