Delivery Systems for Pulmonary Gene Therapy

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Abstract

Delivery of therapeutic genes to the lungs is an attractive strategy to correct a variety of pulmonary dysfunctions such as cystic fibrosis, alpha-1 antitrypsin deficiency, pulmonary hypertension, asthma, and lung cancer. Different delivery routes such as intratracheal instillation, aerosol and intravenous injection have been utilized with varying degrees of efficiency. Both viral and non-viral vectors, with their respective strengths and weaknesses, have achieved significant levels of transgene expression in the lungs. However, the application of gene therapy for the treatment of pulmonary disease has been handicapped by various barriers to the delivery vectors such as serum proteins during intravenous delivery, and surfactant proteins and mucus in the airway lumen during topical application of therapeutic genes. Immune and cytokine responses against the delivery vehicle are also major problems encountered in pulmonary gene therapy. Despite these shortcomings much progress has been made to enhance the efficiency, as well as lower the toxicity of gene therapy vehicles in the treatment of pulmonary disorders such as cystic fibrosis, lung cancer and asthma.

The lung constitutes an important organ for the delivery of therapeutic genes, and one of the most widely investigated. The vast surface area of the lungs can be targeted both for correction of primary pulmonary diseases, and as a potential site for the production of therapeutic gene therapy products for systemic applications. The large network of blood vessels in the lungs, coupled with easy accessibility through the pulmonary airways, enables the lungs to be targeted by both intravenous and topical routes. The latter fact makes the lung unique compared with other organs, allowing specific lung sites such as alveolar cells and bronchial epithelium to be exclusively targeted for different therapeutic applications. Different sites in the bronchi, such as the

proximal and distal bronchial airways, can be targeted with aerosols utilizing the appropriate particle size. The proximal and distal airways could also be accessed through direct injections via fiberoptic bronchoscopy and intrabronchial instillation. Vectors used for the delivery of gene therapy have to contend with natural barriers in the lung such as ciliated epithelium, surfactant proteins, mucus and alveolar macrophages. The alveolar lung parenchyma and endothelial cells can also be targeted through intravenous injection of the delivery vectors. These vectors have to evade the serum proteins and blood immunological responses to transfect the target cells efficiently. In diseased and injured lungs, the barriers for vector delivery of genes can be formidable. Issues such as the degree of transfection of the pulmonary cells and the persistence of gene expression also need to be addressed for effective pulmonary gene therapy.

1. Delivery Vectors

1.1 Viral Vectors

The molecular biology of viruses makes them ideal carriers for gene delivery. Their high transduction efficiency and tissue trophicity are major advantages for gene delivery. Viruses are the most widely used vectors to date in clinical gene therapy protocols. However, the inflammatory and immune responses of the host against these viral vectors are a major handicap, and different strategies to reduce these adverse events have been reported.

1.1.1 Retroviruses

Retroviral vectors, unlike many other vectors, have the ability to integrate into the host genome and stably transduce the cells, which is a likely prerequisite for long-term gene expression. Retroviruses are useful for *ex vivo* gene transfer in stem cell gene therapy and for *in vivo* applications such as treatment of cancer and HIV. The retrovirus is an RNA virus consisting of two copies of a single stranded RNA genome. The genome has different sequences known as *pol*, *gag*, and *env* encoding different structural and catalytic proteins, and is surrounded by a glycoprotein envelope. Although commonly used in gene therapy protocols, retroviruses have their shortcomings. One potential hazard is the production of replication competent viruses that can infect humans. Replication competent retroviruses have been shown to induce malignant T cell lymphoma in immunocompromised monkeys,[1] but virus-induced malignancy or infection have not been reported in humans. Also, integration of the virus into the host genome could potentially inactivate tumor suppressor genes or activate oncogenes, which may lead to a malignant phenotype of the target cell.[2] Retroviral vectors have a limited capacity for inserting DNA (7 to 13Kb) and entail high costs in quality control and

large-scale production. Furthermore, most of the initial retroviruses tested were not able to transduce non-dividing cells. However, retroviruses have been utilized for delivery of genes to airway epithelial basal and secretory cells[3] and also for therapeutic studies in various disease models[4,5] including clinical trials in cancer.[6] Selective targeting of the retrovirus, either cellular or transcriptional, could enhance the efficiency and safety of the vector.[7]

Lentiviruses such as HIV-1 can replicate in non-dividing cells, and are currently being investigated for various gene therapy protocols.[8-10] The best targets for lentiviral vectors seem to be the nervous system, retinal cells and the lympho-hematopoietic system. Lentiviruses show promising application for *ex vivo* transduction of the cells. However, lentiviruses have resulted in rather poor transduction of liver, muscle and lungs.[9]

1.1.2 Adenoviruses

Adenoviral vectors have been the most extensively utilized viral vectors for gene therapy. The adenovirus has an icosahedral shape with the outer capsid comprising of hexon (II), penton base (III) and knobbed fiber proteins (IV). The viral genome has terminal proteins attached to the 5′ termini which has inverted terminal repeats (ITRs). The adenoviral DNA transcription has an early and a late phase. The transcription starts with a complex series of splicing events of various gene cassettes termed E1, E2, E3 and E4.[11,12] Being a respiratory virus, the adenovirus has evolved over years to evade the pulmonary barriers and transfect the bronchial airways. The adenovirus can only accommodate about 2Kb of foreign sequences in its genome. Several deletion adenoviruses have been developed to enhance the packaging capacity of the adenovirus and to reduce its toxicity. The first generation adenovirus vectors were developed by deletion of E1 and/or E3 cassettes [11] making it possible to insert about 6.5 to 7Kb foreign DNA. The E2 sequences, ITR and packaging sequences were left intact. The second-generation adenovirus vectors were developed by removing the E2 genes.[12,13] Some other virus genes and sequences were deleted to make third generation vectors, also known as 'gutless' or high capacity helper-dependent adenovirus vectors.[14] Only the ITR and packaging sequences were retained and the vectors required helper viruses for propagation. The gutless vector does not induce acute liver toxicity, inflammation or cellular infiltration compared with E1-deleted vectors.[15] Further modifications through the Cre-lox helper dependent system has made it possible to prevent the packaging of the helper viruses.[16] Another modification reported recently involved mutations in E1 leading to selective replication of the virus in only p53-defective tumor cells.[17] This virus has been commercially formulated as Onyx 015, and several clinical trials

are currently underway using this vector.[18,19] Adenoviral targeting through antibodies and molecular conjugates to different cells has also been reported to enhance gene transfer, and reduce inflammatory responses.[20,21]

One of the limitations of using adenoviruses for pulmonary gene therapy is that the receptors for adenovirus are located at the basolateral surfaces, making it difficult to target the apical surface of the epithelium through the airways. Modifications to alter the receptor affinity of the virus by engineering the knob fiber capsid proteins[22] and opening the epithelial tight junctions using ethyleneglycol tetra-acetic acid (EGTA), to make the basolateral surface more accessible,[23] are being investigated to enhance the uptake of adenovirus via the airway route. Delivery of adenoviral vectors in perfluorochemical (PFC) formulations, which can diffuse throughout the pulmonary tissue, has been demonstrated to enhance epithelial cell uptake and gene expression.[24]

1.1.3 Adeno-Associated Virus

Adeno-associated virus (AAV) is a non-pathogenic single stranded DNA virus. The replication (*rep*) gene is under the control of p5 and p19 promoters. Since the adenoviruses cannot integrate into the host genome, formation of hybrid vectors with AAV ITRs have been developed to facilitate transgene integration.[25] Also AAV can transfect non-dividing cells, but gene expression in these cells is usually less than that achieved in dividing cells. AAV gene delivery results in long term expression and does not induce high inflammatory responses. However, their small insert size (upper limit of 4.7Kb size DNA) is a major limitation. Another limitation is the establishment and maintenance of packaging cell lines, and the production and purification of the vectors. Recently some of these problems have been addressed by using the HIV long-terminal repeats (LTR) promoter to drive the *rep* gene, resulting in a 10-fold increase in packaging efficiency.[26] Also cell populations containing rescuable AAV recombinant genomes have been developed to enhance packaging.[26] Yan et al.[27] recently demonstrated that the small packaging size of the AAV can be overcome by splitting the insert gene across two different AAV vectors and using trans-splicing to express the gene. AAV vectors have generated a lot of interest in pulmonary gene therapy, especially due to their potential use in cystic fibrosis gene therapy. Flotte et al.[28] demonstrated that endobronchial instillation of AAV expressing the normal cystic fibrosis transmembrane conductance regulator (AAVp5CFTR) could result in transfection of >50% of the bronchial epithelium at the delivery site, although no transfection was detected at other sites. Reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry revealed gene expression up to 6 months after delivery. A subsequent study,[26] showed that intra-

peritoneal injection of AAVp5LacZ resulted in transfection in approximately 25% of the airway epithelial cells. Conrad et al.[29] demonstrated the safety and feasibility of delivering AAV-CFTR to the lungs of rhesus macaques. The vector mRNA was detectable by RT-PCR for up to 6 months post-instillation of the vector, using fiberoptic bronchoscopy. Furthermore, the safety of the vector was determined by various parameters such as pulmonary functions and chest radiographs. An interesting report recently demonstrated that AAV serotype 5 (AAV5), and not AAV2, binds to the apical surface of the epithelial cells, enhancing gene transfer to the bronchial epithelium.[30] This could make AAV5 ideal as gene delivery vehicle for diseases such as cystic fibrosis, and warrants further investigation.

1.2 Non-Viral Vectors

As pointed out in the previous section, one of the major limitations of using viral vectors is the undesirable, and occasionally toxic, immune response to the vectors which makes multiple applications difficult. The advantage of using non-viral vectors over their viral counterparts lies in their cost effectiveness, commercial availability and lack of immunogenicity. However, their major disadvantage has been the low transfection efficiency *in vivo* compared with the viral vectors. Cationic liposomes and cationic polymers are two of the most widely used non-viral vectors for gene delivery.

1.2.1 Cationic Liposomes

The cationic liposomes take advantage of the plasma membrane lipid structure for fusion and delivery of the gene into the cells. Most cationic liposomes consist of a hydrophobic lipid group capable of forming and stabilizing a bilayer, and a cationic lipid group capable of interacting with the anionic DNA. A neutral lipid is often used as a helper or fusigenic lipid for enhancing the transfection efficiency of the liposomes. The exact mechanism of cellular uptake of cationic liposomes and delivery of DNA to the nucleus remains to be elucidated.[31] Significant progress has been made in increasing the transfection efficiency using liposomes. Felgner and Ringold[32] were the first to propose the use of cationic liposomes for gene delivery. The first such reagent consisted of the cationic lipid N-[(1-(2-3-dioleyloxy)propyl)]-N-N-N-trimethylammonium chloride (DOTMA), and was shown to be efficient for transfection of both DNA and RNA into mammalian cells, including suspensions and hybridomas. Soon several other cationic lipid formulations were reported to have significantly higher transfection efficiency,[33] with 3 beta [N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium (DOTAP) showing promising results in therapeutic studies.[34,35]

Some of the early studies proposed the use of cholesterol for increasing the liposome-mediated transfection,[36] with the understanding that cholesterol, being a natural sterol, would enhance the fluidity of the cellular membranes thereby increasing the transfection efficiency. The neutral phospholipid dioleoylphosphatidylethanolamine (DOPE) also increased the transfection efficiency by destabilizing the endosomes, and helping the DNA to escape the endosomes and subsequently reach the nucleus.[37] Different strategies such as stabilizing the liposomes by extruding, demonstrated by Templeton et al.,[38] PEGylation, demonstrated by Hong et al.,[39] and adding protamine, as reported by Li et al.[40] have been shown to increase transgene expression in the lungs after intravenous delivery. Another lipid formulation termed GL67 (an amphiphile consisting of a cholesterol anchor linked to a spermine head group) developed by Lee et al.[41] has been reported to lead to high levels (about 1000 fold increase over naked DNA) of transgene expression in the lungs after intranasal and aerosol delivery. Similarly, the cationic lipid bis(guanidinium)-tren-cholesterol (BGTC) complexed to neutral lipid DOPE has been reported to lead to high levels of gene expression in the lungs after instillation and aerosol delivery.[42,43] Targeting of liposomes using monoclonal antibodies has also been shown to increase transfection of cells.[44] However, serum has been shown to decrease transfection efficiency, probably because of the presence of nucleases that can degrade the DNA, and the presence of proteins in the serum that can neutralize the net positive charge, thereby changing the physical state of the complexes. In addition, instillation as well as intravenous delivery of cationic liposome-DNA complexes leads to induction of inflammatory and cytokine responses leading to decreased levels and persistence of transgene expression. [45,46]

1.2.2 Cationic Polymers

The high charge density and buffering capacity of the cationic polymers helps in compacting the DNA efficiently and protecting it from degradation by nucleases in the low pH endosomal/lysosomal compartment. These polymers have a positive zeta (ζ) potential, a diameter of approximately 40 to 250nm and a high buffering capacity between pH 5 and 7. There are three different types of cationic polymers commonly used for the delivery of genes to cells and tissues. Dendrimers are a class of polymers where the amine material is repeatedly substituted at its amino termini resulting in a branched structure. The extra layer is termed a generation, giving rise to the terminology of dendrimer generation-3, -5, -6, etc. Haensler and Szoka[47] were the first to report the potential use of these polymers for transfecting cells. They demonstrated that a plasmid encoding a luciferase reporter gene gives high transfection in CV-1 fibroblast cells at an optimum charge ratio of 6 to 10 with generation-6 dendrimers. However, these dendrimers were toxic to cells and their toxicity increased when complexed to DNA. Chloroquine 50mM and diethylaminoethyl (DEAE)-dextran 0.5mM increased the transfection efficiency of these dendrimers, but dimethylsulfoxide (DMSO) did not have any effect.[48] Enhanced expression in the presence of chloroquine indicated increased uptake of the dendrimer-plasmid complexes through the endosomes, and DEAE-dextran was postulated to alter the nature of the complexes leading to enhanced uptake. A sub-class of these dendrimers is the 'fractured dendrimers'[49] which are the active components of the heat-treated dendrimers. They have enhanced transfection efficiency because of increased flexibility which in turn decreases the possibility of DNA-dendrimer particle aggregation.

Polyaminoacids are another class of polymers used for efficient transfection of cells both *in vitro* and *in vivo*. Poly-L-lysine (PLL) is the most extensively used polymer of this group, and optimum expression is obtained at a lysine to DNA nucleotide ratio of 1.[50] The PLL-DNA particles are spherical in shape with a diameter of 170nm when complexed in 1.5 M NaCl. PLL stabilizes the A:T rich regions of the DNA, compared with poly-L-arginine (PLA), which stabilizes G:C regions. Both PLA and PLL have similar binding specificities for DNA. The polymer binds to one of the DNA grooves with the basic group of lysine interacting with the DNA phosphate group. PLL, PLA and poly-L-ornithine (PLO) have been shown to give high transfection levels *in vitro* and in the different tissues such as lungs, liver, and kidney after intravenous delivery.[51,52] However, these polyaminoacids, especially the higher molecular weight polymers (>25KDa), have been shown to be quite toxic.

Another group of cationic polymers that have been extensively used for gene therapy are the polyethylenimines (PEI), a synthetic derivative of polyamines. Bousiff et al.[53] were the first to demonstrate PEI mediated transfection of cells *in vitro* and *in vivo*. They also showed that PEI nitrogen : DNA phosphate (N : P) ratios of 7 : 1 to 13.5 : 1 gave the highest rate of transfection. Goula et al.[54] optimized the PEI nitrogen : DNA phosphate ratios, and showed that ratios of around 10 : 1 gave maximum transfection in the lungs after intravenous delivery. They also investigated different molecular weight PEI, both linear and branched forms, and demonstrated that the lower molecular weight PEI (around 25 KDa) gave higher levels of gene expression. However, it should be noted that intravenous delivery of branched PEI (25 KDa) is associated with adverse events such as pulmonary inflammation, hemorrhage, and even death in animals. Bragonzi et al.[55] optimized ratios for intranasal instillation and intravenous delivery to the lungs, and also compared the efficacies of PEI and cationic lipids in mediating systemic gene delivery to the lungs.

They demonstrated that the highest transfection rates in the lungs were achieved by PEI administered intravenously or by intranasal instillation. Our group have also demonstrated the use of PEI for aerosol delivery of DNA for pulmonary gene therapy.[56,57] We have reported anti-tumor effects via aerosol delivery of PEI-p53 complexes in lung cancer models,[58,59] the use of aerosol delivery of PEI-DNA complexes for genetic immunization,[56] and lack of toxicity after PEI-DNA aerosol delivery.[57,60] The mechanism of PEI-mediated gene transfection has also been studied.[61] The buffering capacity of PEI in the endosomes seems to play a crucial role in protecting the DNA and carrying it to the nucleus. This is in contrast with the cationic liposomes, which dissociate from the DNA in the endosomes. In contrast, PEI escorts the DNA to the nucleus.[61] Furthermore, lung surfactants do not affect the transfection efficiency of PEI *in vitro*.[62]

2. Naked DNA

The use of naked DNA for gene delivery has been restricted mostly to direct injection into accessible sites, such as muscle and brain, which would increase the probability of gene expression. Naked DNA for gene therapy of the lungs has not been widely investigated, primarily due to the fact that viral and non-viral vectors result in much higher expression in the pulmonary tissue. The naked DNA lacks the receptor-mediated uptake that is possible with viral vectors. The endocytosis pathway, which the cationic lipids use for transfection, results in degradation of the naked DNA in the low pH milieu of the endosomes/lysosomes, in the absence of protection from non-viral vectors. However, there are a few studies showing that naked DNA can result in similar levels of transfection in the lungs compared with cationic liposomes after instillation delivery.[63,64] Intravenous delivery of naked DNA normally results in poor transfection levels because of interaction with serum proteins and degradation by nucleases.

3. Delivery Strategies

3.1 Instillation

Intratracheal or intranasal instillation takes advantage of the accessibility of the lungs through the respiratory tract. The vector-DNA complex can be applied into the nose or instilled through an intratracheal incision in anesthetized animals, and is inhaled and diffuses into the pulmonary tissues. However, apart from being inherently invasive, the strategy does not distribute the material uniformly throughout the lungs, localizing it randomly through the epithelium in the upper airways as demonstrated by Meyer et al.,[63] Uyechi et al.[65] and Bout et al.[66] After instillation, the transgene localizes mainly in the trachea and mainstream bronchi, with the left and accessory lobes of the lung expressing significantly higher levels than the right lobe of the lung. Another interesting, and rather intriguing, finding was that naked DNA resulted in similar expression compared with cationic lipid-DNA complexes, in contrast to other reported studies.[63,64] The persistence of gene expression was found to be about a week. Instillation is limited by the fluid volume that can be administered safely, and instilled liquids typically distribute according to gravity. Another shortcoming is that the animals have to be anesthetized before delivery of gene vectors through nasal or tracheal instillation. Also, the instillation delivery strategy leads to a localized inflammation and irritation in the lungs, and a small portion of the solution also gets swallowed into the gastrointestinal tract.[63] Cytokine and inflammatory responses to delivery vectors, leading to decreased expression as well as low persistence of gene expression, have also been documented.[46] Despite these limitations, gene delivery by instillation has shown promise in different disease models of endobronchial pulmonary cancer, as demonstrated by Blezinger et al.[67] and Zou et al.,[68] and is also potentially useful for gene therapy in cystic fibrosis as demonstrated by Griesenbach et al.[69] This can be attributed to the fact that a large amount of DNA can be applied directly to the target cells leading to a therapeutic response. The strategy seems to be best suited for application of therapeutic genes to the nasal and upper airway epithelium, which is targeted most efficiently by instillation.[63]

3.2 Aerosol

Aerosol delivery of genes takes advantage of the airways to directly target the lungs. However, unlike instillation, aerosol delivery is relatively non-invasive, and distributes the vector-DNA particles uniformly throughout the pulmonary tissue, including the terminal and respiratory bronchioles. Despite these advantages and successful reports of airway transfection after aerosol delivery,[70] the strategy has not been widely utilized because in some cases there is degradation of DNA during nebulization.[71] Mostly, cationic liposomes complexed to plasmid DNA have been nebulized for aerosol gene therapy and have demonstrated significant levels of transgene expression in the lungs.[72-75] Viral vectors have also been utilized for aerosol delivery of genes to the lungs, resulting in high transfection efficiency.[76] However, the inefficiency of the aerosol delivery system would require large amounts of vector, which could be rate limiting for viruses. Also, Pillai et al.[77] demonstrated plasmid stability and airway transfection after aerosol delivery of cationic lipid-DNA formulations using an ultrasonic nebulizer. Recently we have demonstrated that PEI , a cationic polymer, could protect the DNA dur-

ing nebulization and achieve higher levels of transfection in the lungs after aerosol delivery compared with different cationic lipids tested.[56] The levels of transgene expression were sufficient to achieve an anti-tumor response in two different lung cancer models[58,59] and resulted in a robust antibody response against the protein product of the gene used for genetic immunization.[56] Furthermore, the aerosol PEI-DNA does not lead to significant acute inflammation[57] or induction of pro-inflammatory cytokines such as interleukin (IL)-1β or tumour necrosis factor (TNF)-α in the lungs or the serum.[60]

Modification of aerosol output particle size as well as nebulization conditions can help target different regions of the bronchial airway tree specifically and uniformly.[78] Large particle size aerosols (>4μm) target mainly the oropharyngeal layers of airways, whereas small aerosol particles (<2μm) can penetrate into the peripheral pulmonary tissues such as terminal and respiratory airways.[79] Similarly, modification of pulmonary functions such as tidal and minute volumes by breathing low carbon dioxide levels (about 5% CO_2) can also help deposit a higher percentage of aerosol particles into the lungs, leading to increased transgene expression.[57] Chu et al.[23] demonstrated that pretreatment of airways with EGTA can enhance the transfection and expression of aerosolized or instilled viral and non-viral formulations. The hypothesis is that EGTA opens up the tight junctions in the bronchial epithelium, enhancing the uptake of the aerosolized particles from the basolateral surface of the cells.

The major limitation for aerosol delivery of gene therapy formulations could be due to compromised airways as occur in pulmonary diseases and injured lungs. Airways that are constricted due to mucus or inflammation could result in decreased transfection by the aerosol particles, and macrophage clearance of the vectors could also be enhanced.[80] However, using mucolytic agents can facilitate mucus clearance in the airways, thereby enhancing deposition and transfection of subsequently aerosolized formulations. Altered breathing parameters of patients could also influence the deposition and transfection of the aerosol particles. The inherent inefficiency of the system is another issue, requiring large amounts of material for significant transfection in the lungs.[70]

3.3 Intravenous

There are situations when mucus, surfactant proteins or disease pathology present impenetrable barriers to the delivery of vectors administered via the airway route. Also, there are complications when the desired target sites include the interstitium and lung alveolar and endothelial cells rather than the airway epithelium, as might be the case with metastatic disseminated tumors and pulmonary hypertension. The major advantage of using the intravenous route is that the vascular endothelium can be specifically targeted and a large amount of DNA can be administered in a single injection. The mesh-like network of blood vessels in the lungs also help to distribute the complexes to a large surface area. Although biodistribution after intravenous delivery is not specific to the lung[38,55] delivery by this route is still able to achieve high levels of transgene expression in the lungs. The gene expression can be made specific for the lungs by using tissue-specific promoters driving the transgene. Liu et al.[81] have studied extensively the different parameters influencing liposome-mediated intravenous gene delivery, including, lipid : DNA ratio, comparison of different neutral lipids, efficacies of small unicellular vesicles (SUV) and multi-lamellar vesicles (MLV), and particle size of the liposomes. However, Li et al.[45] demonstrated that cytokine induction [such as IL-1β, TNF-α, and interferon (INF)-γ] against the non-viral delivery vectors (cationic liposomes) is a major disadvantage for intravenous delivery of cationic lipid-DNA complexes. Cytokine induction leads to decreased transgene expression, low persistence and a refractory period between consecutive doses. Immune and cytokine responses are important concerns related to intravenous delivery of viral vectors, especially because they can induce global toxicity.[82] Different viral vectors have been modified by deletion of viral replication elements such as E1A, as well as envelope glycoproteins, to reduce these undesired effects. Targeting of the endothelium with anti-platelet endothelial cell adhesion molecule (PECAM) antibodies have been proposed to enhance uptake and transgene expression in the lungs after intravenous administration, and to reduce toxicity.[44] Also, Orson et al.[83] have developed albumin-polyethylenimine-DNA (MAA-PEI-DNA) complexes that lead to increased transfection in the lungs after intravenous delivery. The transfection after intravenous delivery is mostly restricted to the endothelial and alveolar cells. Although the intravenous mode of delivery has been tested in an attempt to target the epithelium, this has not been very successful.

3.4 Direct Injections into the Disease Site

In complications such as a solid tumor mass or cystic fibrosis lungs with mucus, where administration via the respiratory tract or intravenously may not be successful, direct fine needle injections using bronchoscopy or computer tomography–assisted injections can be made to the disease sites.[84,85] The major advantage of this approach is the high degree of transfection at the injected site. However, it is a complicated and invasive technique and multiple injections at different sites are required for effective treatment, making it cumbersome for patients and clinicians. To

date, only viral vectors have been utilized for this strategy because of the fact that the viruses generally have much higher transfection efficiency after direct injection, compared with non-viral vectors. The direct inoculation strategy has been mostly utilized for treatment of lung cancer, mesothelioma and cystic fibrosis, where specific regions of the lungs can be selectively targeted.[84-87]

4. Current State of Gene Therapy for Pulmonary Diseases

4.1 Cystic Fibrosis

Cystic fibrosis is a classic example of a lethal autosomal recessive genetic disease. It is characterized by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, resulting in absence or dysfunction of the cyclic adenosine monophosphate (cAMP)-regulated chloride channel and imbalanced chloride and sodium transport across the apical surface of the epithelial cells.[88] This dysfunction in ion transport results in a lethal phenotype with the airways being clogged with mucus and sputum. Data from cell culture studies suggest that transfection of just 5 to 10% of the affected epithelial cell monolayer with wild type *CFTR* may be enough to produce a therapeutic effect in patients with cystic fibrosis by normalization of the trans-epithelial chloride transport.[89] Since the cloning of the *CFTR* gene in 1990, several gene therapy strategies have been applied to deliver the gene to the epithelium,[90,91] with approximately 20 different studies in various phases of clinical trials. Most of the studies have been Phase I clinical trials, with either viral or non-viral vectors administered intranasally or by aerosol. Both functional (potential difference across epithelium, ion conductance) and molecular (PCR analysis of vector transcripts) markers were studied, along with an analysis for safety. Knowles et al.[92] initially demonstrated that nasal application of adenoviral vector to nasal epithelium produced a very low degree of gene transfer and minimum functional correction. Alton et al.[93] demonstrated modest phenotypic correction in patients, as detected by chloride ion conductance, after exposure to cationic liposome vectors encoding CFTR via aerosol. However, there was no improvement in sodium transport function in the lung. Harvey et al.[94] sprayed adenoviral vector encoding CFTR into a small area in the bronchial airway, and detected transcripts by RT-PCR at levels >5% above the endogenous mutated transcripts in the transduced cells. In this study, transcripts were detected up to 3 days post administration of the virus, although no levels were detected after 30 days. Furthermore, repeated administration of the virus resulted

in loss of expression, although this did not correlate with systemic anti-adenovirus neutralizing antibodies.

The thick sputum and mucus in the airways has been another difficult barrier to surpass for aerosol delivery.[95] One strategy proposed to overcome this is to use perfluorochemical (PFC) liquids along with delivery vectors which diffuse throughout the lungs and can help transfect distal regions of the pulmonary tissue.[24,96] Another strategy is to use mucolytic agents such N-acetylcysteine, or DNAse, which helps to clear the sputum,[97] before gene delivery to the lungs. The use of calcium chelating agents also helps in opening up the airway epithelial cell tight junctions, and allowing the vectors to access the receptors on the basolateral surface of the cells.[23,98] Zuckerman et al.[84] used bronchoscopy for injecting adenovirus-*CFTR* vector into the disease site of patients with cystic fibrosis, thus overcoming the mucus barrier to the delivery vector. An alternative approach to topical delivery of genes to the airways is through the intravenous route, a strategy being extensively investigated (see section 3.3).[55,69,99] Although it has resulted in some airway expression,[69,99] the intravenous route of administration has its own barriers consisting of endothelial cells, alveolar cells and epithelial basolateral membrane, before the vectors can reach the apical epithelial cells.

4.2 Lung Cancer

Lung cancer is the single largest cause of deaths among cancer patients. The lung is also a primary site of tumor metastases, and inhibition of lung metastases is a major clinical challenge. The mortality rate is very high, despite extensive chemotherapy, irradiation and surgery. The advent of gene therapy has raised hopes for a possible cure for lung cancer, and lung cancer is the most extensively studied among all gene therapy protocols. One of the major challenges in designing gene therapy studies for cancer is the polygenic nature of the disease, with significant environmental influences. In contrast to cystic fibrosis, where CFTR is known to be dysfunctional, genetic analysis of cancer presents a far more complicated picture. Two distinct approaches have been followed for gene therapy of lung cancer. One gene therapeutic strategy for lung cancer is designed around the *p53* and retinoblastoma (*Rb*) tumor suppressor genes, with replacement to correct the cellular functions or lead the cell into programmed cell death. Aerosol, intravenous and instillation approaches have been utilized in animal models with *p53* and *Rb* genes, using both viral and non-viral vectors.[58,59,100-102] Another approach centers on using immunomodulatory genes such as those encoding interleukin (IL)-12, IL-4, IL-2, and interferon (INF)-α,[103-105] to marshal the body's innate immune system

against the tumor cells. Also, suicide genes such as thymidine kinase have been utilized in gene therapy for increasing drug sensitivity and tumor suppression.[106] Another interesting approach that has been postulated in recent years is to target tumor-induced angiogenesis,[107] or new blood vessel formation, thereby depriving the cancer cells of nutrients leading to their slowed growth, and possible death. Different anti-angiogenic factors such as angiostatin, endostatin and thrombospondin[108-110] as well as the *p53* gene[111] have been shown to result in potent anti-tumor responses by inhibiting tumor-induced blood vessel formation. Viral vectors are the most commonly used delivery vectors because of their high transduction efficiency, although non-viral vectors have also shown promise.

In their Phase I clinical trial, Schuler et al.[112] demonstrated the safety of administration, and also the stabilization of local non-small cell lung cancer, in patients administered adenovirus containing the *p53* tumor suppressor gene. Weill et al.[87] recently demonstrated partial response in 25% of patients and alleviation of bronchial constriction in 50% of patients treated with adenovirus-*p53* vector injected bronchoscopically into tumors, causing bronchial constriction. The patients received an injection once every 28 days with no evidence of acute toxicity. In lung cancer patients, Roth et al.[6] demonstrated a very potent anti-tumor effect of p53 delivered using retroviruses by direct intra-tumor injections using bronchoscopy. They further demonstrated that combination of *p53* gene therapy with DNA damaging drugs, such as cisplatin, results in enhanced tumor suppression.[113] The hypothesis proposed is that the drugs trigger DNA damage and *p53* then diverts the cell into apoptosis resulting in cell death. Similarly, combination of γ-irradiation with *p53* expression results in enhanced cell death in tumors.[114] Bronchial epithelium is the most common site of primary tumors such as bronchoalveolar cell carcinoma, and delivery of gene therapy vectors via the airway route may be of therapeutic benefit in the treatment of these tumors. Mesothelioma is another disease that can benefit by direct injections and airway transfection with therapeutic genes. Although gene therapy alone shows promise, a multi-modality approach might be more suitable for polygenic diseases, such as lung cancer.

4.3 Apha-1 Antitrypsin Deficiency

Alpha-1 antitrypsin (AAT) protein is an endogenous antiprotease, produced primarily in the liver, which counteracts the activity of the proteases and elastases produced from inflammatory cells. The protein reaches the lungs through the systemic circulation after being secreted into the blood stream from the liver. Chronic lung inflammation and pulmonary emphysema are the primary manifestations of the disease in AAT-deficient patients. Conventional therapy is intravenous administration of purified AAT protein. However, the high expense and the frequent administration of the protein emphasize the need for alternative therapies. Delivery of the *AAT* gene encoded in a delivery vector has several advantages. Firstly, it can localize the transgene expression to the lungs leading to an enhanced local level of the AAT protein. Secondly, the long term expression of AAT in the lungs would help to reduce the frequency and cost of administration. Thirdly, gene therapy may reduce the potential hazards patients might face in the case of injection of AAT protein, which is purified from pooled human serum. A significant amount of data has been collected recently to propagate the idea of *AAT* gene therapy. Most of the delivery vectors used are non-viral vectors, presumably because viral vectors could aggravate the already inflamed lungs despite achieving higher levels of transgene expression.[115] Aerosol and intranasal administration are theoretically better delivery systems since they can target the bronchial epithelium of the lungs, leading to a localized secretion of the AAT protein. Rosenfeld et al.[116] were the first to show that intranasal delivery of adenovirus encoding the *AAT* gene to the airway epithelium of cotton rats resulted in robust gene expression in the lungs. Brigham et al.[117] have shown that significant levels of AAT expression can be achieved in the nasal mucosa of AAT-deficient patients after intranasal delivery of cationic liposomal formulations of the *AAT* gene. They further demonstrated the advantages of such therapy over conventional protein therapy and characterised by decreased inflammation, increased local AAT levels and long-term expression of AAT. Canonico et al.[118] have demonstrated the efficient transfection of the rabbit airway cells by *AAT* gene delivered by the aerosol and intravenous routes. The PEI-DNA aerosol delivery system developed and optimized by our group[57] also holds potential for *AAT* gene therapy, given the fact that the aerosol particles can very efficiently transfect the airway epithelial cells leading to a long term (about a month following a single aerosol exposure) transgene expression in the lungs.[119] Adult respiratory distress syndrome (ARDS) and emphysema, two pulmonary diseases that are caused by unregulated neutrophil responses, can also be potentially treated by *AAT* gene therapy that can inhibit the action of neutrophil elastase.[117]

4.4 Asthma

The pathogenesis of asthma is not clearly understood. The disease is triggered in part by environmental factors. A number of other factors have been proposed [120] but there is still no consensus on the pathways and factors involved in triggering airway hyperresponsivenss and inflammation characteristic of asthma.

The cell types involved in the pathogenesis of asthma are another point of debate. A variety of cells have been implicated including macrophages, lymphocytes and bronchial epithelial cells.[121] Given multiple potential targets, gene therapy for asthma is a complicated proposition and still theoretical at best. However, considerable advances have been made and interesting studies have raised the hope for future gene therapies for asthma. Most of the gene therapy strategies proposed involve the modulation of T helper type 2 (Th2) cytokines produced by the lymphocytes, which play a central role in the pathogenesis of asthma. Nyce and Metzger[122] demonstrated the use of antisense DNA in down regulating the Th2 cytokine, IL-4, in an animal model of asthma. Another strategy is to over express INF-γ and IL-12 which inhibit the secretion of proinflammatory cytokines, such as IL-4, as demonstrated in animal models of asthma.[123,124] Delivery of CpG oligonucleotides, associated with the regulation of the costimulatory factors B7.1 and B7.2, is another strategy proposed to reverse Th2-associated allergic airway responses.[125] Ohkwara et al.[126] demonstrated that intranasal delivery of recombinant adenoviral vector containing IL-10 can reduce the total cell count and eosinophil numbers, as well as IL-5 levels in the bronchial alveolar lavage fluids in a murine model of allergen-induced airways inflammation. Mathieu et al.[127] reported that transfection of A549 human lung epithelial cells with a glucocorticoid receptor repressed transcription factor NF-kB and AP-1 activities even in the absence of corticosteroids. This novel finding may present an alternative therapeutic strategy to control asthma in patients whose asthma is resistant to treatment with corticosteroids. For asthma gene therapy, delivery of genes using non-viral vectors may be more suitable than using viral vectors because viral vectors can potentially induce inflammatory responses, further aggravating the hyperactive airways. Aerosol delivery of gene therapy vectors could be the delivery system of choice, since it can efficiently target the airway epithelium,[119] a primary disease site in asthma. The long-term expression of transgenes in the lungs may also help to reduce the cost and frequency of treatment.

5. Conclusions

Gene therapy holds tremendous therapeutic potential for a variety of pulmonary diseases. Most of the preliminary work regarding the genetic basis of diseases and development of vectors and delivery systems has been extensively investigated with numerous studies underway. The next phase of development in gene therapy would be to enhance the efficiency of transfection and lower the toxicity of the delivery vectors. In the immediate future, gene therapy could be used more as an adjunct to conventional therapies. However, there is little doubt that it could be standard therapy for pulmonary diseases in the next few decades.

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