Gene therapy for pulmonary disease has attracted a great deal of attention since the first report of successful gene delivery 10 years ago. Potential indications for gene therapy include chronic illnesses such as cystic fibrosis and \( \alpha_1 \)-antitrypsin deficiency, and acute illnesses such as acute transplant rejection and chemotherapy-induced lung injury. The key technological impediment to successful gene therapy is vector optimization. Viral vectors, including adenovirus and adeno-associated virus, have relatively low efficiency in vivo. In addition, adenovirus has been associated with a brisk inflammatory response and limited duration of expression in the lung. Nonviral vectors, particularly liposomes, have also been tried, with limited expression efficiency and some toxicity. Although work is ongoing to improve adenoviral and adeno-associated viral vectors and test other viral and nonviral vectors, an ideal vector has not yet been identified. Several important barriers to successful gene therapy, including the host inflammatory response, promoter down-regulation, tissue-specific targeting, and physical barriers to gene delivery in the airway, will need to be overcome. Despite these daunting problems, several human gene therapy trials have been completed, using adenovirus, adeno-associated virus, and liposomes. In general, these trials have been focused on safety, and have shown that there is dose-dependent inflammation in response to adenovirus. Adeno-associated virus appears to cause little inflammation. Demonstration of successful gene delivery and transcription has been quite variable in human trials. In general, the level of expression of transgene appears to be quite low. In summary, although there is great promise for gene therapy in the lung, significant challenges remain in translating this technology to successful human therapy.

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Key words: adeno-associated virus; adenovirus; DNA; inflammation; plasmid; vector; virus

Abbreviations: AAT = \( \alpha_1 \)-antitrypsin; AAV = adeno-associated virus; AdV = adenovirus; CAR = Coxsackie adenovirus; CF = cystic fibrosis, CFTR = CF transmembrane conductance regulator; HSV = herpes simplex virus; IL = interleukin

Gene therapy has been the subject of a great deal of information, and misinformation, over the past decade. The first proposed use of gene therapy was complementation of single gene defects, such as adenosine deaminase deficiency, cystic fibrosis (CF), and the like. Soon after, the potential for gene therapy to treat acquired diseases such as cancer and cardiovascular disease was pursued. After an initial period of enthusiasm and expectation, the reality of the complexity, and potential risks, of the endeavor has become apparent. Despite the growing awareness of the inherent difficulties in translating gene transfer into viable treatment for human disease, there is also great reason to be optimistic about the prospects for successful gene therapy in the next decade. The purpose of this review is to summarize progress made to date in the area of pulmonary gene transfer. Potential indications for gene therapy, vectors, barriers to gene delivery, and status of clinical trials will be reviewed.

**Indications for Gene Therapy**

CF is the pulmonary disease that has received the most interest as a target for gene therapy. In some respects, CF appears to be an ideal gene therapy candidate. The disease is the result of mutations in a
single gene (CF transmembrane conductance regulator [CFTR]), and the principle target is the airway epithelial cell, which is in theory accessible to gene delivery. However, as will be discussed below, airway epithelial cells have proven to be a difficult target for gene therapy.

A variety of other lung diseases have been identified as potential gene therapy targets.\(^5\) \(\alpha_1\)-Antitrypsin (AAT) deficiency is another prevalent lung disease resulting from a single gene mutation. Like CF, AAT deficiency has as a potential target the airway, although more distal gene delivery may be beneficial in AAT deficiency. Whereas both CF and AAT are associated with concomitant liver disease, the prevalence of severe liver failure is much higher in AAT deficiency. In addition AAT is a secreted product of the liver associated with concomitant liver disease, the prevalence of severe liver failure is much higher in AAT deficiency. Therefore, gene delivery and correction directed toward hepatocytes has received attention in AAT deficiency.\(^6\)

Acquired lung diseases may also be candidates for gene therapy. For instance, primary pulmonary hypertension may be amenable to gene therapy, even though \(< 15\%\) of cases are familial. Lung cancer is another likely candidate for gene therapy.\(^7\) Another very intriguing candidate for gene therapy is lung transplantation. Prevention of the acute phase of reperfusion lung injury as well as short-term and long-term rejection may be targets for gene transfer. Although a long way off, it is also possible that prevalent diseases with a complex genetic basis, such as emphysema and asthma, and idiopathic disorders such as pulmonary fibrosis may ultimately be candidates for gene therapy.

### Gene Therapy Vectors

Both viral and nonviral vectors have been proposed for pulmonary gene therapy. Each has advantages and disadvantages. Adenovirus (AdV) was the first viral vector studied in the context of lung gene therapy. In a landmark article, Rosenfeld and co-workers\(^8\) demonstrated that AdV could be used to deliver AAT to the airway epithelium of cotton rats. Before using the AdV as a gene therapy vector, several modifications to the virus must be made. First, to prevent replication of the virus some, or all, of the native viral DNA must be deleted. Initial (first-generation) AdV vectors had the E1 region of the AdV genome removed, and thus are referred to as \(\Delta E1\) AdV vectors. As products of the E1 gene are required for the initial phase of viral replication, this was believed to be sufficient to eliminate replication. The gene of interest was then mated with a promoter to drive expression, and this construct was inserted into the missing E1 region. To allow packaging of the recombinant DNA into viral particles, a packaging cell line in which the missing E1 genes were inserted into the native DNA of the cell was used.

This method has proven very effective at producing high titers of AdV vectors encoding a variety of gene products, including CFTR and AAT. However, as more experience was gained in working with AdV vectors, it became apparent that even without the E1 region, a small amount of viral genes were still being transcribed. This led to a robust host inflammatory response that limited the utility of the vector. To overcome this limitation, many modifications to the viral genome have been made. Deletion of additional genes in the E4 region seems to increase persistence of expression and reduce inflammation in some animal models.\(^9\) Recently, high-titer production of an AdV vector with no endogenous viral genes (so-called gutless or helper-dependent vectors) has been reported. These vectors have the theoretical advantage of producing no viral antigens, and thus little potential for an inflammatory response. In theory, this will result in prolonged expression and increased ability to re-treat subjects without the interference of neutralizing antibodies and cellular immunity.

A limitation of AdV vectors that cannot be easily overcome with reengineering is the lack of integration. Integration means that the viral genome inserts itself into the host chromosome, leading to stable, long-term expression. For genetic disorders such as CF and AAT deficiency, this could be a great advantage. In early gene therapy studies, a modified retrovirus called Maloney virus was used as an integrating vector. This worked very well in proliferating cells in culture, but was very ineffective in cells with low replication index and in \textit{in vivo}. Although it is possible that retrovirus could be useful for gene transfer to the developing lung \textit{in utero}, it is not a likely vector for gene transfer to the adult lung. However, another integrating virus, adeno-associated virus (AAV) may be useful.\(^10\)

AAV gets its name from an obligatory requirement for concomitant AdV (or herpes simplex virus [HSV]) infection to allow replication \textit{in vivo}. However, it is completely unrelated to AdV in its structure. It is also a DNA virus, but its genome is much smaller (4.7 kilobase vs 36 kilobase for AdV). The native virus integrates into a specific site in chromosome 19. Initial studies with AAV demonstrated its ability to be used to transfer DNA into airway epithelial cells \textit{in vitro} and \textit{in vivo}. However, because of the small size of the genome, it was necessary to remove all viral genes except the inverted terminal repeats on the ends. After this modification was made, the virus lost its ability to produce selective integration.
It remains somewhat uncertain whether recombinant AAV vectors integrate randomly, or simply remain as stable episomal DNA (ie, extrachromosomal). In either case, it appears that expression for several months or longer is possible with AAV. Another early limitation of AAV was the difficulty in producing high-titer vector. However, in the past year or two, this problem has been overcome, and high-titer, human-grade vector can now be produced. A final limitation of AAV that cannot be easily overcome is the small insert size. For diseases such as CF, this is a significant problem, as the CFTR gene itself completely fills the vector, and additional regulatory elements necessary to drive and modulate expression cannot be added. Recombinant AAV administration does result in the formation of neutralizing antibody, which could limit the ability to retreat with the same vector. However, in contrast to AdV, AAV appears not to produce a significant cellular inflammatory response, and thus it remains a good candidate for lung gene therapy.

Other viral vectors have been proposed for use in gene therapy, including HSV and lentivirus (of which HIV is a member). HSV appears to be particularly useful for gene delivery to neural tissue, and could be used to transduce nerves in the lung. Lentivirus, like Maloney virus, is an integrating retrovirus. Unlike Maloney virus, lentivirus can infect and integrate into the DNA of nonreplicating cells. This was first demonstrated for CNS tissue, but could be useful for lung gene therapy as well. However, feasibility of HSV and lentivirus for pulmonary gene therapy has not been demonstrated.

Nonviral vectors have also been proposed for pulmonary gene therapy. Cationic lipids have been the best studied. The concept of using cationic lipids is that anionic DNA plasmids can be mixed with cationic lipids to produce a complex that can then fuse with cell membranes and allow the new genetic material to enter the cytosol. Once the DNA enters the cytosol, it must then be transported to the nucleus, where transcription can occur. The transfer of plasmid to the nucleus is a very inefficient process, which is rate-limiting for nonviral gene delivery. In contrast, viral vectors such as AdV take advantage of translocation mechanisms triggered by the viral capsid, greatly increasing efficiency.

The composition of lipids in liposomes varies widely and is critical to vector targeting and efficiency. Additional components, such as protamine and proteins, can both increase efficiency and promote selective targeting to the lung. An interesting feature of cationic liposomes is their ability to target the lung circulation after IV injection. Many, although not all, lipid–DNA complexes are preferentially taken up and expressed in the pulmonary endothelium. This does not appear to be a first-pass phenomenon, but rather the result of specific interactions between the complexes and the lung microvascular endothelium.

Other nonviral DNA delivery systems have been described, including polymers and small synthetic beads. Even naked DNA diluted in hypotonic fluids can produce some gene delivery to lung epithelium. However, the utility of these delivery systems, particularly in vivo, has not yet been proven. For all of these systems, it appears that gene expression efficiency is quite low. For diseases such as CF, this may be a major limitation, whereas for other diseases, low-level expression may be adequate.

**Inflammatory Response to Gene Delivery**

As mentioned above, a major limitation to gene delivery has been the host inflammatory response. This has been particularly well studied for AdV. Multiple related and redundant host defense mechanisms are activated by AdV vectors. Preformed antibodies are the initial line of defense, and vary depending on the serotype and prior infection history of the host. There is also an antigen-nonspecific cytokine response, including interleukin (IL)-6 and tumor necrosis factor-α, which is triggered by the vector and may produce local or systemic inflammation. Cellular immunity, including CD8+ cytotoxic lymphocytes and CD4+ helper lymphocytes, is also induced. The former leads to rapid clearance of transfected cells, and the latter, to generation of antibodies that prevent subsequent re-treatment with the same vector.

As noted above, one approach to limiting inflammation in response to AdV vectors has been to remove more viral genes from the vector. An alternative approach has been to use pharmacologic manipulation of the host immune response to mitigate the inflammatory response. Pretreatment with corticosteroids, cyclophosphamide, IL-12, and certain monoclonal antibodies can limit the inflammatory response, at least in animal studies.

A major hope in the use of nonviral vectors was to cause less inflammation. Although there appears to be much less specific immune response, nonspecific inflammation in response to lipid-DNA complexes is a major problem to both intravascular and intracheal delivery. After intracheal delivery, induction of a brisk, neutrophil-rich, inflammatory lesion is seen. This response is associated with induction of IL-6, tumor necrosis factor-α, and interferon-γ. The response can be reduced by elimination of abnormal methylation sites in bacterial sequences in
the plasmid DNA. However, there also appears to be a response to the cationic lipid component of the complex as well.

Intravascular delivery of lipid-DNA complexes also induces lung inflammation. This response is characterized by release of large quantities of Th1 cytokines from lung and spleen mononuclear cells. This appears to lead to induction of cytotoxic T lymphocytes and rapid elimination of transduced cells. In mice deficient in the Th1 cytokine interferon-γ, transgene expression is much greater, suggesting the inflammatory response contributes to reduced transgene expression.

**OTHER BARRIERS TO LUNG GENE THERAPY**

The immune response is a major barrier to gene therapy. However, the airway presents other, unique problems. For AdV vectors, the human airway has developed a number of protective strategies. A very important component is a lack of expression of the native Coxsackie adenovirus (CAR) receptor on the apical surface of airway epithelial cells. The lack of this receptor greatly limits the infectious potential of AdV. Several strategies are being investigated to circumvent this problem. Transient reduction in cell-to-cell junctions with calcium phosphate coprecipitation allows access of the vector to the basolateral membrane where the CAR receptor is located. Another strategy is to change the affinity of the vector to other ligands. The AdV vector has a protein capsule that includes two components, the fiber knob and penton base, which participate in CAR receptor binding. By mutating these proteins, affinity for the CAR receptor can be reduced or eliminated.

Further alteration of the fiber knob can redirect tropism to other receptors expressed on the apical membrane of epithelial cells. Another significant barrier to gene delivery in the diseased airway is mucus. A variety of components of mucus can bind to AdV and prevent its association with receptors. It is possible that combination with mucolytics could augment gene transfer efficiency. Another interesting possibility is the use of perfluorocarbons as an adjuvant to vector delivery. For genetic diseases such as CF, it is also possible that initiation of gene therapy earlier in the course of the disease will reduce this problem.

**BEYOND GENE THERAPY**

Gene therapy has as its central principle the addition of gene function through gene transfer. For a disease like CF, this means inserting a functional copy of the CFTR gene into airway epithelial cells that are genetically defective at this locus. However, there are some situations where even if this were accomplished, the disease phenotype would persist. An example of this is AAT deficiency. Although lung gene delivery of the AAT gene might prevent the progression of obstructive lung disease, it would do nothing to prevent the fatal hepatic failure affecting up to 25% of individuals. This liver failure is the result of misfolded protein in the Golgi apparatus and endoplasmic reticulum of hepatocytes. Buildup of these proteins leads to cell injury and organ failure.

An alternative strategy being investigated is gene correction therapy. The theory behind this strategy is to insert a molecular mechanism into cells that targets the abnormal DNA sequence in the chromosome, and corrects the mistake. Two techniques have been used to do this: RNA-DNA chimeras and ribozymes. The concept being used is to target the repair construct to the regions flanking errors in the native gene. RNA-DNA chimeras then take advantage of endogenous DNA repair enzymes to fix the resulting mismatch. Ribozymes effect similar repair to the aberrant DNA enzymes. These strategies require the delivery of very high concentrations of the construct for a brief period to the affected organ. In this regard, the liver is an ideal target, and studies in animals have demonstrated feasibility of the approach. It remains to be seen whether this approach can be used to correct the genetic error in patients with AAT deficiency.

**STATUS OF HUMAN TRIALS**

The vast majority of human gene therapy trials for lung disease have focused on CF, and results have been mixed. More than 20 trials using AdV, AAV, and cationic liposomes have been completed. In all cases involving lung delivery, the study design was phase I (safety and dosing). To summarize the results of many trials, one can say that AdV has dose-specific and vector-specific toxicity in the form of lung inflammation. Some individuals in CF AdV trials have exhibited transient pulmonary infiltrates. This appears to be dose-dependent, appearing at higher vector concentrations. Most subjects also exhibited increased titers of neutralizing antibodies. It has been suggested that rotation through various serotypes of AdV viruses could allow repeated dosing even in individuals who have developed neutralizing antibodies. All trials used early generation AdV vectors. Trials with more highly engineered AdV vectors have not yet been reported.

Aerosol delivery of lipid-DNA complexes was
studied in several trials. In general, there appears to be less toxicity, although, as noted above, localized inflammation and induction of Th1 cytokines can be seen. Data from AAV trials are sparse at this time, but toxicity also appears to be low.

In an effort to obtain some information about efficacy, a strategy of delivering the vector to the nasal epithelium was developed. This has the advantage of transducing a readily accessible tissue, while exposing the study subject to little risk. Unfortunately, results of multiple trials have been unimpressive. Several trials have demonstrated by the very sensitive polymerase chain reaction technique successful transfer of CFTR gene. However, electrophysiologic demonstration of correction of the functional defect has generally been absent or very modest.

**CONCLUSION**

Many genetic and acquired lung diseases are potential targets for gene therapy. The initial enthusiasm associated with gene therapy has now been replaced by recognition of the great difficulties inherent in this endeavor. Several viral and nonviral vectors have been demonstrated in preclinical studies to be suitable for pulmonary gene delivery. However, problems with efficiency and host immune response remain to be solved. Given the rapid progress the field has made, it is likely that clinical application of gene therapy to pulmonary diseases will become a reality in the next decade.

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