28 Gene Therapy for Lung Diseases

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SUMMARY

Gene therapy is under development for a variety of lung disease, both those caused by single gene defects, such as cystic fibrosis and α_1-antitrypsin deficiency, and multifactorial diseases such as cancer, asthma, lung fibrosis, and ARDS. Both viral and nonviral approaches have been explored, the major limitation to the former being the inability to repeatedly administer, which renders this approach perhaps more applicable to conditions requiring single administration, such as cancer. Progress in development and clinical trials in each of these diseases is reviewed, together with some potential newer approaches for the future.

Key Words: α_1-antitrypsin deficiency; ARDS; asthma; cystic fibrosis; fibrosis; lung cancer; lung inflammation; lung transplantation; pulmonary edema.

INTRODUCTION

At first sight, given both the accessibility of the airways and the genetic basis of certain pulmonary diseases, the lung seems an ideal candidate for gene therapy. Several routes of access are available, including topical administration to the airway, intravascular administration, and direct injection. Two respiratory diseases, cystic fibrosis (CF) and α_1-antitrypsin (α_1-AT) deficiency, are common single gene disorders for which the genetic basis is known, and for which treatment strategies are not curative. These inherited diseases are the obvious initial target for gene therapy, but it has become clear that nongenetic and acquired diseases including cancer may also be amenable to this approach. Following early optimism, a wide range of barriers has emerged to gene expression in both the airway and alveolar region, some of which are of limiting success. The background to these barriers, methods being employed to overcome them, and the clinical progress in CF, α_1-AT deficiency, lung cancers, and a variety of other pulmonary diseases are discussed.

VECTORS FOR GENE TRANSFER IN LUNG DISEASE

Although naked DNA is capable of transfecting cells, low levels of efficiency have led investigators to explore the use of vectors, systems designed to transport the DNA into the cell. The majority of those in clinical trials have been based on either a recombinant virus or a nonviral system such as liposomes or polymers. Each approach has advantages and disadvantages (Thomas, 2003; Niidome, 2002); although a detailed discussion of each is outside the scope of this chapter.

VIRAL VECTORS Modified, replication-deficient adenoviruses (Ad) have been by far the most widely used of the viral vectors, despite the fact that their receptor (coxsackie adenovirus receptor) is not available from the apical side of the cell, but is situated basolaterally (Walters, 1999). Attempts to overcome this problem are discussed later. Adeno-associated virus (AAV) is a small replication-defective virus. In naturally acquired infection, which occurs in the presence of adenoviral coinfection, the AAV inserts into a specific site in the host genome, providing the potential advantage, therefore, of increased duration of transgene expression. However, this integration probably does not occur with the current generation of recombinant viruses (Snyder, 1999). In any case, opinion is divided as to whether this would be desirable, unless it could be targeted to a quiescent area of the genome rather than being random. An additional drawback is the small size of the virus, which makes insertion of the large pieces of cDNA, such as cystic fibrosis transmembrane conductance regulator (CFTR), problematic. Retroviruses also integrate, although this is not normally site-specific, but certain of these, such as lentiviruses have the added advantage of transfecting nondividing cells (Quinonez, 2002). The paramyxovirus, Sendai virus possesses several advantages, such as rapid penetration of mucus, binding to the cell surface via its apically situated receptor, sialic acid, and the capacity for cytoplasmic expression, which overcomes the potential of the nuclear membrane as a barrier. Based on these advantages, one group is exploring this virus as a vector for gene transfer in CF (Yonemitsu, 2000). A common drawback of all viral vectors is the host immune response. Recognition of viral proteins leads to both inflammation at the time of administration and to significantly reduced efficacy of any subsequent application, largely related to the development of neutralizing antibodies (Yei, 1994). Attempts to combat these mechanisms have included removal of various parts of the viral genome (Boucher, 1994) and the coadministration of anti-inflammatory or immunosuppressive agents (Chirmule, 1999; Kolb, 2001) although the safety of the latter approach remains to be confirmed. The requirement for repeated application will depend on which disease is being targeted. Clearly, for certain cancers, a single injection may suffice, and in fact, a strategy of transfecting cells in order to render them immunogenic and trigger cell killing is being utilized in some cancer trials (Tursz, 1996). In contrast, however, in a life-long disease such as CF, repeated application will almost certainly be required. In such a context, a viral vector may be inappropriate.

NONVIRAL VECTORS Many synthetic vectors are being explored for gene therapy; those used clinically have mainly been cationic liposomes or synthetic polymers. The full details of the

mechanism by which the cationic liposome delivers DNA into the cell are incompletely understood, but the concepts have been well reviewed (Lee, 1996; Nishikawa, 2001). As much of the DNA delivered in this fashion fails to reach the nucleus, the system, at least theoretically, may be less efficient than some viral vectors. However, this does not appear to have been the case in clinical trials. More importantly, the relative lack of toxicity or immunogenicity is a major benefit. As discussed later, however, these approaches are not completely free from adverse effects. Nanoparticles, short pieces of DNA, are compact, small diameter particles, which may facilitate their transport through the nuclear pore. Addition of a receptor to mediate cellular uptake, serpin enzyme complex receptor, has shown promise with this approach in animal models (Ziady, 2002). Efficiency of nonviral gene transfer methods may be enhanced by a variety of adjunctive physical interventions including electroporation (Taylor, 2003; Sumner, 2003) or complexing the vector with magnetic particles and applying a magnetic field (Plank, 2003). There are clearly advantages and disadvantages to both the liposomal and the recombinant viral approaches. Future systems may be designed to incorporate the best of each.

ROUTES OF ADMINISTRATION

The route of administration depends on the desired cellular distribution and function of the therapeutic protein. Topical administration, directly onto the airway surface, would seem most appropriate for epithelial membrane-bound proteins such as CFTR, or proteins to be secreted into the airway, such as α_1-AT or cytokines. Such airway epithelial expression is likely to be most easily achieved by inhalation or nebulization, both of which have been extensively studied for delivery of nongene-based drugs. Some gene transfer studies have used bronchoscopic instillation. Although this could be impractical for diseases requiring repeated application such as CF, it may be useful for endobronchial tumors, in which direct visualization and targeted application would be of benefit. Localized injection, for example, into a tumor or pleural space may be highly appropriate for certain cancers, although limitations arise when only a small proportion of the tumor is accessible endobronchially, or when metastatic spread has already occurred. Intravascular injection may be appropriate to target the alveolar epithelium. For diseases such as α_1-AT deficiency or certain inflammatory diseases, in which the desired protein is secreted and circulates systemically, gene transfer to a distant organ, such as muscle or liver, which could serve as a "factory," is an attractive option.

BARRIERS TO GENE EXPRESSION IN THE AIRWAY

Limited success in early studies of gene transfer to the lung and airway led to the recognition of a wide range of barriers, both to cell entry and gene expression. An understanding of these mechanisms is beginning to lead to the rational design of either novel vectors, of adjunct therapies directed at the barrier itself, or of nondirected physical delivery methods. A series of barriers can be considered during the passage of a vector from outside the host through to the nucleus of the desired cell. With topical application to the lung, the vector must evade the mucociliary clearance system, penetrate the mucus barrier (which may be made more difficult by certain disease states, e.g., CF) (Stern, 1998) enter the cell through the glycocalyx and avoid lysosomal degradation to reach the nucleus, whereupon it must cross the nuclear membrane. Although the systemic approaches or local injection may help to overcome certain of these early barriers, the latter ones clearly still

pose a challenge. Additionally, for application by any route, the fact that both the vector and the DNA are composed of foreign material renders the complex potentially immunogenic. The vector, the DNA, or the expressed transgene product could trigger a host response, which could result in elimination of the applied complex, death of the expressing cell, or both. Details of the identified barriers in this journey and the methods being attempted to overcome these are described in detail.

CELL ENTRY: TOPICAL ADMINISTRATION

Reaching the Cell Surface   The conducting airways are lined with both ciliated and mucus-secreting epithelial cells, the major integrated function of these being the rapid removal of inhaled material (Houtmeyers, 1999). The mucus barrier also serves to prevent direct contact between inhaled particles and the cell surface, which in the case of airborne infection, for example, serves to limit the epithelial inflammatory response to pathogens. These successful host defense strategies are clearly potentially detrimental for topical gene transfer. In certain disease states this problem may be even more pronounced; for example, CF sputum, which is abnormal both in volume and composition, inhibits gene transfer by both viral and liposomal vectors (Stern, 1998; Perricone, 2000) as may pre-existing nonspecific inflammation at the airway surface (van Heekeren, 1998). Recognition of these barriers may lead to the pursuit of adjunctive therapies to increase efficacy, some such approaches such as the use of recombinant human DNase (Stern, 1998) or Nacystelyn (Ferrari, 2001) to reduce mucus viscosity, have shown promise in preclinical studies. Finally, several investigators have explored the use of either surfactant (Raczka, 1998) other low surface tension liquids (Weiss, 1999) or thixotropic agents (Seiler, 2002) to facilitate spreading of gene transfer vectors throughout the respiratory tract and increase contact time with the airway surface. These compounds may increase the distal airway and alveolar expression of therapeutic genes at the expense of more proximal airway expression, so the target area needs to be clearly identified before this approach could be considered applicable. The cell surface glycocalyx also inhibits entry; with one study demonstrating significantly enhanced Ad-mediated reporter gene expression after the removal of sialic acid with neuraminidase (Pickles, 2000).

Entering the Cell   Some vectors require a specific cell surface receptor in order to enter the cell. For example, although Ad naturally infects respiratory epithelia, the coxsackie adenovirus receptor to which it binds is not expressed on the apical cell surface. In vitro studies have demonstrated increased gene transfer after breakdown of intercellular tight junctions (Parsons, 1998), permitting access to basolaterally situated receptors, although the clinical applicability of such approaches remains to be determined as this could lead to potentially deleterious loss of epithelial integrity. Other groups are developing an interest in pseudotyping of viruses, adding a ligand specific for a cell surface receptor, in an attempt to increase efficiency (Romanczuk, 1991). With an alternative approach, the efficiency of both Ad and AAV has been increased by coprecipitation of these vectors with calcium phosphate, which appears to increase nonreceptor-mediated uptake via the apical cell surface, but may also aid endosomal release (Fasbender, 1998; Walters, 2000). One advantage of vectors such as Sendai virus is the direct cell entry via membrane fusion, which bypasses the endosome, allowing the virus to avoid degradation (Yonemitsu, 2000).

CELL ENTRY: SYSTEMIC ADMINISTRATION   Attempts have been made to circumvent barriers at the mucosal surface by delivering transgenes systemically. The intravenous route might make it possible to access the basolateral membrane of airway epithelial

cells, characterized by a higher rate of endocytosis and an increased density of viral receptors. However, most studies have demonstrated that the cells transfected via the systemic circulation are pulmonary endothelial cells or alveolar epithelial cells (Griesenbach, 1998), with only a minority reporting airway epithelial cell transfection (Zhu, 1993; Koehler, 2001). This is likely to be related to a number of barriers including reticuloendothelial system clearance, escape from the vasculature, the interstitium, and the epithelial basement membrane. Various attempts to overcome these barriers have been reviewed (Fenske, 2001; Niidome, 2002).

CROSSING THE CYTOPLASM Once inside the cell, lysosomal breakdown (Zabner, 1995) and destruction by cytoplasmic DNases (Lechardeur, 1999) may further limit gene expression (Duan, 2000). Preliminary data with AAV suggest that the use of proteasome inhibitors may improve gene expression, and another group has demonstrated that specific sugar moieties conjugated to nonviral vectors may influence endosomal escape (Grosse, 2002). Alternative approaches include the addition of peptides to facilitate cytoskeletal transport, or the breakdown of cytoskeletal elements such as microtubules or microfilaments (Kitson, 1999).

ENTERING THE NUCLEUS In contrast to the situation with rapidly dividing cells, such as those grown in vitro, in nondividing or slowly dividing cells in vivo, the nuclear envelope successfully prevents the entry of foreign material into the nucleus. Molecules that need to enter, for example, transcription factors, do so through the nuclear pore complex, mediated by nuclear localizing sequences. This barrier is likely to be a major limiting step in successful gene transfer. In attempts to increase nuclear entry, investigators have explored the addition of a variety of moieties, for example, peptide sequences from the HIV TAT protein (Snyder, 2001) and novel nuclear localizing sequences (Munkonge, 1998). Alternatively, this barrier can be overcome completely by the use of vectors, which do not require nuclear entry for gene expression, such as Sendai virus or the development of cytoplasmic expression systems such as those incorporating T7 promoter and RNA polymerase (Brisson, 1999).

THE HOST IMMUNE RESPONSE The duration of gene transfer can be limited by a variety of mechanisms including transcriptional silencing of the promoter, or loss of the transfected cell, either naturally or via host defense recognition. Regarding the former, some progress has been reported with human polyubiquitin, UbC (Gill, 2001) or UbB promoters (Yew, 2001). Both innate and adaptive immune responses can limit the efficiency and duration of gene transfer, and may pose problems with all routes of administration, including production of a secreted protein at a distant site. Within the respiratory tract, alveolar macrophages can act either directly as phagocytes or indirectly as antigen presenting cells, resulting in fairly rapid clearance of a topically applied gene transfer vector (Worgall, 1997). Similarly, macrophages in the reticuloendothelial system can mediate clearance of a systemically applied vector (Plank, 1996). If vectors are not cleared by such innate immune mechanisms, the adaptive immune system has the potential to significantly impair gene transfer and expression. Both cell-mediated and humoral responses have been demonstrated with viral or synthetic vectors although the major problems in clinical trials have been encountered with viral vectors. Initiation of a cytotoxic T-lymphocyte response may result in killing of the transduced cells, thus effectively limiting the duration of transgene expression. Recognition of viral coat proteins and the production of neutralizing antibodies have created problems in some trials of repeated application of viral-mediated gene transfer (Yei, 1994), and naturally acquired anti-Ad antibodies in the sputum of CF patients have been shown to inhibit transduction (Perricone, 2000). Selective deletions in the viral genome, and coadministration of agents to reduce the cytotoxic T-lymphocyte response (Chirmule, 1999), are showing some promise in increasing the duration of gene expression. However, this problem is not limited to viral vectors. The presence of unmethylated CpG motifs on plasmid DNA has been suggested as a cause for an observed inflammatory response with nonviral vectors (Schwarzt, 1997; Yew, 1999). Efforts are being aimed at either selectively methylating such motifs (although this can reduce expression levels) (McLachlan, 2000) or inhibiting the pathways through which they signal with agents such as chloroquine (Yew, 2000). Unlike the situation with viral vectors, cationic liposomes are equally effective on subsequent application (Hyde, 2000), which may render them more useful for diseases requiring repeated administration. Although apparently not a significant problem in clinical trials, the possibility of antibody production to previously unencountered transgene-derived protein also exists.

SPECIFIC LUNG DISEASES

CYSTIC FIBROSIS CF results from a variety of mutations in the gene encoding the CFTR protein, a cAMP-regulated chloride channel, leading to abnormal ion transport at the apical surface of epithelial cells (Welsh, 1993). Pulmonary disease dominates the clinical picture, leading to death in more than 90% of patients (Foundation, 1995). The hallmarks of CF lung disease are early, severe, and sustained neutrophil-mediated inflammation, and persistent infection (Armstrong, 1995). Conventional treatment for CF patients has advanced greatly, but at best, it slows the inevitable progression of lung damage. New approaches are therefore required.

Gene Therapy Although other organs are affected in CF, the lung is the major site of pathology, and thus has been the target in the majority of gene therapy trials. Topical gene delivery to the airway epithelium aims to normalize the functions of CFTR including ion transport. There are several unresolved questions with this approach, including (1) which types of cell should be transfected, (2) what degree of correction is required, and (3) how success should be measured. With respect to the cell type targeted, maximal CFTR expression in non-CF airways appears to be in the submucosal glands (Engelhardt, 1992), although clinically detectable disease begins in the distal small airways, in which expression in the surface epithelium is lower (Engelhardt, 1994). Topical application, for example via inhalation, is likely to target the surface epithelium, but is less likely to reach the deeper submucosal gland cells. Whether gene transfer to these cells will be necessary to elicit a clinical effect remains to be determined. Furthermore, surface epithelial cells are terminally differentiated (Warburton, 1998); loss of the transgene therefore occurs with the eventual death of the cell. Interest is focusing on identification of respiratory stem cells, gene transfer to which may have the potential to effect long-term CFTR expression in progeny cells. Regarding what degree of transfection is required; this is made more complex by the realization that different levels of expression may be required to restore the various functions of CFTR. For example, lower numbers of cells need to be corrected to restore chloride transport compared with those required for normalization of sodium absorption (Johnson, 1995), and differences have also been observed regarding the correction of glycoconjugate sulfation and ion transport (Zhang, 1998). Which function(s) of CFTR are most important and whether all identified functions (and perhaps as yet unrecognized ones) need to be corrected is an important question that remains to be resolved. However, based on

genetic studies, it would seem that as little as 5–10% of wild-type levels of CFTR in each cell might be sufficient for a normal disease-free phenotype (Gan, 1995). The final issue is one of the assessment of success. Detection of either mRNA or CFTR protein provides evidence of gene expression but no confirmation of functional correction. Ion transport was the first function of CFTR identified, and is thought to be key in the disease pathophysiology. As such, it is the function most commonly assessed in both gene-based and pharmacological clinical studies. Ion transport is assessed most readily in vivo by measurement of transepithelial potential difference (PD), both at baseline and in response to a variety of drugs, including those that block the sodium channel, such as amiloride, and that stimulate chloride secretion (Knowles, 1981; Middleton, 1994). These measurements can be obtained in the airway via the broncho-scope (Alton, 1999), as well as from the nasal epithelium. Additional ex vivo techniques such as epifluorescence microscopy have also proved useful in some studies (Stern, 1995), and alternative functions of the protein, such as those involved in bacterial adherence (Alton, 1999), have also been utilized as end points.

Clinical Studies

Viral Vectors   Many studies conducted in the nose and the lower airway, particularly those in the lower airway, have relied on molecular rather than functional evidence of gene expression. Expression has been transient, with variable dose responses. In one of the earliest studies, Ad/CFTR was administered to both the nose and lower airway bronchoscopically (Crystal, 1994). One of the highest dose patients became unwell with fever, hypoxia, and pulmonary infiltrates. The problem was attributed to vector-induced inflammation as no infective virus was detected. Significant local inflammatory reactions and the stimulation of neutralizing antibodies was a feature of the high-dose arm of another nasal study (Boucher, 1994), and flu-like symptoms and a cell-mediated immune response were seen at a high vector dose in a broncho-scopic study (Zuckerman, 1999). Interestingly, a study from France found no acute toxic effects at doses up to 5.4×10^8 plaque-forming units, and no increased inflammatory parameters or antiviral antibodies in bronchoalveolar lavage or serum (Bellon, 1997). Molecular analysis confirmed successful gene transfer, although no functional end points were employed. Attention has focused on the potential problems with repeated administration, with clinical trials addressing this issue in the nose and in the lower airway. In the nasal study, despite there being no detectable adverse effects, the ability of the Ad/CFTR to correct the abnormal chloride transport was reduced on subsequent application (Yei, 1994). In the lower airway study, Ad/CFTR was administered via an endobronchial spray in three doses over a 9-mo period to patients with CF, with bronchoscopic assessment 3 and 30 d after administration (Harvey, 1999). Almost 3 d after the first administration, vector-derived mRNA was detected in a dose-related manner, with those in the high-dose group expressing levels in the 5% of wild-type range, which has been demonstrated in vitro to be sufficient for phenotypic correction. Examination of distribution was unfortunately not possible, as the vector could not be identified with fluorescent *in situ* hybridization. mRNA levels were undetectable in all patients by d 30. The second administration resulted in some nondose-dependent expression, but the third administration produced no expression in any sample. Similarly to the study previously reported, these investigators detected no increase in serum neutralizing antibodies, although gene expression was not seen in patients with high preexisting levels of antibody. This study, therefore, confirms, that although repeated administration of adenoviral vectors appears safe, efficacy

is severely compromised, although the exact mechanism for this is uncertain. AAV-mediated *CFTR* transfer has been assessed in CF patients using preexisting antrostomies into the maxillary sinus for ease of administration and assessment (Wagner, 1998). Ten patients received escalating doses in an unblinded fashion with no significant inflammatory response. Molecular end points demonstrated gene transfer, with DNA detected up to 41 d after administration, but assessment of expression was difficult. Functional assessment with PD responses to isoprenaline and amiloride demonstrated some changes, although numbers were small. This group then performed a phase II study using a similar technique, which did not show significant changes in PD or time to sinusitis exacerbation, but in which there was some evidence of changes in inflammatory parameters (Wagner, 2002). Safety was confirmed in a phase I study in which AAV-*CFTR* was administered by nebuliza-tion to the lungs of mildly affected patients and the results of a mul-ticenter placebo-controlled trial by the same group have been reported in abstract form (Moss, 2002). Forty-four patients from eight centers were recruited and small but significant changes in both lung function and inflammatory markers were described.

Nonviral Vectors   Several early placebo-controlled clinical tri-als of liposome-mediated *CFTR* gene transfer to the nasal epithelium confirmed safety and demonstrated a degree of functional correction (Sorscher, 1994; Caplen, 1995; Gill, 1997; Porteous, 1997; Knowles, 1998). These studies led us to the first trial of liposome-mediated *CFTR* to the lower airway of patients with CF (Yonemitsu, 2000). Administration was well tolerated, but respiratory symptoms includ-ing mild chest tightness and cough were seen in both groups. This had not been observed with liposome alone in non-CF subjects (Chadwick, 1997), and may relate to the pulmonary inflammation present in the lungs of the CF patients. In addition, all patients in the treatment group reported mild influenza-like symptoms within the first 24 h. The reason for this was unclear, but it may relate to the presence of unmethylated CpG groups on the bacterially derived DNA. Importantly, these symptoms were not reported after nasal administration, which was included for comparison purposes, sug-gesting that for safety at least, the nasal epithelium may not be a good surrogate site for such trials. The major efficacy end point was lower airway PD. In neither group was there any change in the parameters of sodium absorption (baseline or amiloride response). The treatment group however demonstrated a significant response to perfusion with low chloride and isoprenaline, of approx 25% of non-CF values. Unlike the reported problems with readministration of adenoviral-mediated gene transfer, a study using DC-Chol/DOPE reported that repeated nasal administration was well tolerated and could be effective (Hyde, 2000).

α**-1-ANTITRYPSIN DEFICIENCY**   Deficiency of α_1-AT, the principal endogenous antiprotease, leads to pulmonary emphy-sema and in some cases, liver disease. The disease is inherited in an autosomal-recessive fashion, with several mutations having been identified resulting in absent or severely low levels of circu-lating protein (Coakley, 2001). The principal action of α_1-AT in the lung is to counter the adverse effects of proteases such as neu-trophil elastase in the distal conducting airways and alveoli. Therapy involves avoidance of damaging environmental triggers such as cigarette smoke, and symptomatic treatments. Plasma-derived α_1-AT can be administered intravenously, but is costly and has a short half-life necessitating frequent administration (Pierce, 1997). Its purification from human serum also raises the possibility of viral transmission. Gene therapy has, therefore, been considered as an alternative approach.

In contrast to the situation with CF, the secreted nature of the deficient protein makes expression at a distant site a possibility for exogenous gene transfer, and simplifies end point assays. The liver, as the natural site of synthesis, would seem the logical choice, and in vivo studies in animals have assessed several delivery systems and routes of administration including tail and portal vein injections, biliary infusion, and direct intrahepatic injection (Stecenko, 2003). Although successful, these initial studies reported inadequate levels of protein. AAV has also been used to transduce muscle, and one group has reported higher protein levels by implantation of genetically modified myoblasts in mice (Bou-Gharios, 1999). Another approach has been to transduce hematopoetic cells with a retrovirus encoding human α_1-AT, and reinfuse the bone marrow into mice (Saylors, 1998). A postulated advantage of this approach might be high expression of the transgene at sites of inflammation. The treated animals demonstrated extremely low levels of human protein at a 3-wk time-point, after which levels declined.

Several studies have assessed the feasibility of targeting the respiratory epithelium directly, in a fashion similar to that described for CF. In early studies on the cotton rat, adenoviral-mediated α_1-AT gene transfer via the trachea led to the detection of functional protein within epithelial cells for up to a week (Rosenfeld, 1991). Although the cells were capable of secreting the protein, the extracellular levels were once again too low to be therapeutically effective. Since then, studies demonstrating that airway epithelia can secrete the protein both apically and basolaterally provide encouraging evidence that the interstitium can be reached. In a study of aerosolized cationic liposome-mediated α_1-AT gene transfer to the rabbit lung, protein was demonstrated both in the airway and alveolar cells (Canonico, 1994).

The first clinical study of α_1-AT gene transfer has been reported (Brigham, 2000). Patients with α_1-AT deficiency received a single dose of cationic liposome-α_1-AT complex into one nostril, with the other nostril acting as a control. Protein was detected in nasal lavage fluid, with levels peaking at d 5 at approx one-third of normal values. This rise was not seen in fluid from the control nostril. In addition, levels of the proinflammatory cytokine, interleukin (IL)-8, were decreased in the treated nostril. Most interestingly this anti-inflammatory effect was not observed when intravenous-administered purified α_1-AT protein achieved levels within the normal range in nasal lavage, leading the authors to speculate that different routes of administration may lead to a variable response in different sites of expression. Future studies will address this issue and assess administration to the lower airway. One potential difficulty in later-phase studies will be the design of end points to assess clinical benefit in this disease, which progresses extremely slowly in nonsmoking patients.

CANCER   In comparison to the two previous single gene disorders, cancer may seem a less obvious choice for gene therapy. Although cancer is fundamentally a disorder of genes, many mutations of different genes are usually necessary to produce disease. However, this is a rapidly growing field, with several approaches demonstrating some success.

Tumor Suppressor Gene Therapy   Mutation of the tumor supressor gene, *p53*, is one of the commonest findings in certain types of lung cancer (nonsmall cell) (Rom, 2000). Importantly, patients bearing mutations in *p53* on tumor cells are less likely to respond to treatment, either chemotherapy or radiotherapy. Early evidence that *p53* could be a useful therapeutic target came from in vitro studies (Fujiwara, 1994). Transduction with normal *p53*

rendered lung cancer cell lines more susceptible to apoptosis, an effect that was enhanced in the presence of the chemotherapeutic agent, cisplatin. Significantly, in animal models, a bystander effect has been observed, in which a subset of cells expressing the gene inhibits the growth of neighboring nontransfected cells.

Several viral-mediated clinical trials have been reported on patients with demonstrated *p53* mutations who were failing to respond to conventional treatment, although none of these has been controlled (Roth, 1996; Schuler, 1998; Swisher, 1999; Nemunaitis, 2000; Yen, 2000). The gene transfer agent has been administered locally into the tumor either via the bronchus or percutaneously under CT guidance. Side effects have been generally well tolerated, with some evidence of gene expression at high doses. Some studies have reported either stabilization or regression of growth in the injected tumors, with an absence of effect seen at uninjected sites. However, one multicenter study involving 25 patients with nonresectable tumors showed no evidence of benefit over conventional chemotherapy alone (Schuler, 2001).

Suicide Gene Therapy   The basis of this approach is the transfer of a gene encoding an enzyme capable of converting a nontoxic to a toxic chemotherapeutic agent (Smythe, 2000). This limits the activity of the active drug to the site of gene expression and thus minimizes side effects. The most commonly used system has been the herpes simplex thymidine kinase (HSV*tk*) gene, which converts ganciclovir into a triphosphorylated derivative. This is incorporated into DNA in place of guanosine triphosphate and leads to inhibition of cell replication. Two major advantages of this approach are a significant bystander effect owing to local spread of toxic metabolites, and the stimulation of a local immune response. The approach is being utilized in trials of malignancies in many sites, including in patients with pleural mesothelioma (Sterman, 1998). With an adenoviral vector, intrapleural administration of HSV*tk* to 21 patients led to expression of transgene protein in just over 50%. Strong immune responses against Ad were initiated, although this was well tolerated. Partial regression of tumors has been reported in some patients.

Immunotherapy   Immunogenetic therapy is based on the transfer of genes encoding molecules involved in the host immune response in an attempt to enhance immune recognition and destruction of tumor cells. Such genes include various cytokines, interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor, and heat shock proteins (Leroy, 1998). An alternative strategy has been to introduce a foreign gene, such as the bacterially derived β-*galactosidase*, in the hope that cells expressing this gene will be targeted for destruction. Two clinical trials examining feasibility demonstrated local tumor regression and the induction of strong antibody responses both to the adenoviral vector and the transgene (Tursz, 1996; Gahery-Segard, 1997). Other studies have administered vaccinia virus-mediated *IL-2* intrapleurally to patients with mesothelioma (Mukherjee, 2000). No clinical benefit has been shown despite increases in T-cell infiltrates. Preclinical studies are exploring the possibilities of using cytokine cocktails.

Oncolytic Adenovirus   There are several replication-specific Ad, such as ONYX-015, which has a deletion in the E1B-55 kDa gene region that is required for inhibition of wild-type p53 function. Therefore, viral replication is prevented in cells with normal p53 function, but permitted in tumor cells with mutant p53, in which it leads to cell death, and a significant bystander effect (McCormick, 2000). This effect has been confirmed in preclinical studies (Bischoff, 1996; Heise, 1997), with the latter study showing synergy with conventional chemotherapeutic agents. One clinical study

confirmed both safety and feasibility of intravenous-administered ONYX-015 in patients with end-stage metastatic lung cancers (Nemunaitis, 2001). Infusions were administered weekly with or without conventional chemotherapeutic agents. No dose-limiting toxicity was detected at doses up to 2×10^{13} particles, mild adverse events being fevers, rigors, and transient elevation of liver enzymes. All patients showed an increase in neutralizing antibody levels, and raised levels of circulating cytokines. Viral infection of tumor deposits was seen in one patient and at doses above 2×10^{12} circulating viral genome indicated replication. Replication-specific oncolytic viruses may therefore become a useful approach. Future studies may address the effects of transient immunosuppression, reduction in hepatic clearance of virus, and combination of this approach with cytotoxic drugs or therapeutic transgenes.

INFLAMMATORY AND MISCELLANEOUS DISEASES

Acute Lung Injury   Acute lung injury (ALI) (adult/acute respiratory distress syndrome) is the end result of a variety of insults, including severe sepsis, aspiration, trauma, near-drowning, and pancreatitis (Dennehy, 1999). The clinical hallmarks of ALI include impaired oxygenation, which is often poorly responsive to invasive ventilation and patchy infiltrates on chest X-ray in the absence of a raised pulmonary arterial wedge pressure or left atrial hypertension. Despite the improvements in intensive care management, which have led to a reduction in mortality, ALI carries a poor prognosis, particularly in children. No specific therapy exists, and management is largely supportive (Weinacker, 2001). The pathophysiology of ALI is a sequential process of immediate injury, exudative alveolar inflammation with edema and, finally, fibroproliferative repair (Dennehy, 1999). The recognition that the early stages of this process are characterized by generalized intravascular activation, endothelial damage and high levels of proinflammatory cytokines, has led to the development of novel gene and small molecule-based approaches to treatment. These have some theoretical advantages over the use of proteins, including the potential for cell specificity, the possibility of delivering intracellular proteins, duration of action, and possibly cost. Most approaches target either inflammation or oxidative stress, although other investigators have explored the potential for increasing fluid reabsorption and reducing pulmonary edema in this disease process.

Anti-Inflammatory Approaches   As with other inflammatory diseases, inflammation can be targeted either by antagonizing proinflammatory cytokines, or by increasing the levels or effects of anti-inflammatory cytokines. In the context of ALI, studies have been undertaken both with conventional gene transfer techniques, and with antisense oligonucleotides, which reduce translation to protein by specific binding to mRNA. Ad-mediated *tumor necrosis factor* (*TNF*)-α *receptor* gene transfer reduces septic shock in mice injected with lipopolysaccharide (LPS), and reduces pulmonary inflammation (Rogy, 1995). IM delivery of Ad/*IL-10*, a major anti-inflammatory cytokine, showed similar benefits, with a marked suppression of LPS-induced TNF-α and IL-6 production (Xing, 1997). Both *prostaglandin synthase* and *nitric oxide synthase* genes have demonstrated potential therapeutic benefit in animal models of ALI (Conary, 1994; von der Leyen, 1995). Antisense technology has also been used in this context: oligonucleotides against ICAM-1, a major intercellular adhesion molecule, reduced endotoxin-induced neutrophil influx to the lung (Kumasaka, 1996). Hyperoxia has been implicated in lung damage seen in both ALI and infant respiratory distress syndrome. The demonstration that this effect occurs via a mitogen-activated protein kinase, p38MAPK (which also mediates LPS and TNF-α induced damage) (Lee, 1994), has led to another

novel approach. This pathway is attenuated by CO, a byproduct of heme degradation catalyzed by hemoxygenase. Ad-expressing inducible *hemoxygenase*, *HO-1*, instilled intratracheally, increased the survival of rats with hyperoxic lung damage (Inoue, 2001).

Targeting Pulmonary Edema   In the healthy state, alveolar liquid is cleared by the basolaterally situated Na⁺,K⁺-ATPase, which is upregulated during the resolution phase of pulmonary edema (Factor, 2001), a recognized feature of ALI. A variety of vectors have been used to overexpress this ATPase in animal models of lung injury with pulmonary edema, with studies reporting both improved fluid clearance and enhanced survival (Factor, 2000; Stern, 2000). An alternative approach has been to increase activity of the epithelial sodium ion channel, ENaC, the levels and function of which are both upregulated by β(2)-adrenoreceptor (β[2]-AR) expression. β(2)-AR increased alveolar fluid clearance by more than 100% in a rat model (Dumasius, 2001), suggesting that this approach may be applicable in pulmonary edema clearance.

Asthma   Asthma is a disease of high prevalence characterized by type-2 T-helper lymphocyte-mediated inflammation (Lee, 2001) and airway hyperreactivity. Treatment with bronchodilators and anti-inflammatory agents is successful in treating wheeze, cough, and breathlessness in the vast majority of patients (Suissa, 2001). However, for the subgroup who does not respond, novel therapeutic approaches may be relevant. Following success in animal models with Th1-type cytokine protein therapy (IFN-γ [Lack, 1994] or IL-12 [Schwarze, 1998]), beneficial effects have been demonstrated with *IFN-γ* (Dow, 1999), *IL-4 receptor antagonist* (Zavorotinskaya, 2003), and *IL-12* (Hogan, 1998) gene transfer. Thus, in line with the multifactorial nature of the disease, many options are being explored for new gene therapies for asthma. Given the success of conventional treatment however, it is likely that such therapies may only be useful for a minority of patients who do not respond to standard regimes.

Fibrotic Lung Disease   Lung fibrosis can be idiopathic, part of a multisystem (e.g., autoimmune) disorder, or iatrogenic (following radiotherapy or drugs such as bleomycin [Fonseca, 1999]). The prognosis is often poor and available therapies are limited. A variety of growth factors, in particular transforming growth factor (TGF)-β are considered key in the progressive nature of the disease (Sime, 2001), leading to these molecules as targets for novel therapies. Both Smad7, a TGF-β antagonist (Nakao, 1999), and decorin, an endogenous proteoglycan with anti-TGF-β activities (Kolb, 2001) administered intratracheally to bleomycin-treated mice led to a significant reduction in fibrosis. Another group has demonstrated prevention of radiation-induced lung fibrosis and improved survival with liposome-mediated *manganese superoxide dismutase* (Epperly, 1998). The design of radiation-induced promoters (Scott, 2000) may help to limit sites of expression of therapeutic transgenes both in radiation-related fibrosis and lung cancers. Finally, a clinical study has shown dramatic success with IFN-γ 1β protein therapy, which in combination with prednisolone, led to improvements in pulmonary function and oxygen saturation (Ziesche, 1999); in contrast, the group treated with the corticosteroid alone deteriorated. This, and other reports suggesting a role for Fas-mediated alveolar cell apoptosis (Kuwano, 1999), may lead to new gene-based strategies for this disease.

Lung Transplantation   The major obstacle to organ transplantation programs worldwide remains the lack of sufficient donor organs. Medium to long-term success is, however, further limited both by acute ischemia-reperfusion injury, which is particularly problematic for lung transplantation (Mal, 1998), and by the host

response leading to organ rejection (Ward, 2000). Given the shortage of available organs, strategies to attenuate these processes would be of major benefit. Organ transplantation theoretically creates a unique window of opportunity for gene therapy; in addition to administration to the host before removal of the organ, or the recipient after surgery, therapeutic genes could be administered to the organ ex vivo during the procedure. The feasibility of *Ad-β-gal* transfection either before procurement (via tracheostomy) or ex vivo after surgical removal has been demonstrated (Cassivi, 1999). At the critical time of reperfusion, significantly greater transgene levels were shown in lungs transfected via the tracheostomy than in those transfected ex vivo. Levels in other organs were virtually absent, confirming limitation to the lungs. However, another study using liposomes demonstrated that ex vivo transfection was superior to intravenous injection of donors prior to organ harvesting (Boasquevisque, 1999). Another concern may be the cold preservation of organs prior to transplantation, which adversely affects efficacy of gene transfer (Boasquevisque, 1998). The optimal route and timing of gene administration may therefore depend on the vector and the desired function and site of transgene expression.

Various mediators have been implicated in ischemia-reperfusion injury, including stimulated leukocytes and platelets, complement, proinflammatory cytokines, and oxidants (Mal, 1998). Attempts to combat this process with exogenous recombinant proteins have been limited, thought largely to be related to the inability to achieve and maintain high local levels. Gene transfer may therefore be a more useful approach. Ad/*hIL-10* was administered intravenously to rats 24 h before organ harvest (Itano, 2000). When assessed 24 h after isotransplantation, dose-dependent IL-10 expression was observed along with significant improvements in gas exchange and neutrophil sequestration when compared with controls (Ad-*LacZ*). In the higher dose group, myeloperoxidase and NO synthase were also decreased, suggesting that IL-10 may be of benefit in reducing injury at the time of reperfusion. Several groups have reported a reduction in rejection of donor lungs with a variety of methods. Lipid-mediated *Fas ligand* was administered retrogradely through the pulmonary venous system, prior to lung removal along (Schmid, 2000) with a single dose of cyclosporine. Compared with controls, the rats receiving *Fas*-transfected lungs had better d 5 gas exchange, and demonstrated significantly less histological evidence of acute rejection. Other groups have reported success with *TGF*-β both lipid-mediated ex vivo (Mora, 2000) and Ad IM into donor post-transplant (Suda, 2001). However, similarly to the situation with CF and α1-AT deficiency, robust end point assays in lung transplantation trials will be complicated by the clinical heterogeneity of the patient groups studied.

FUTURE DIRECTIONS AND SUMMARY

The principle of gene therapy for lung disease has been proved, with clinical trials showing successful gene transfer in a variety of genetic and acquired disorders. The major problem is with low levels of efficiency, both in terms of cell entry and, for chronic diseases, duration, and the limitation by the host immune response of repeated application with viral vectors. Regarding efficiency, research is ongoing into both overcoming barriers to gene transfer and vector design, including viral pseudotyping and the development of newer generation cationic liposomes and synthetic vectors. Regarding long-term expression, approaches being investigated include manipulation of either the vector or the host to permit repeated administration, the use of integrating vectors, and attempts to identify and target respiratory epithelial progenitor cells. Focused efforts on these areas are likely to lead to the development of successful gene therapy approaches in the near future for a number of respiratory diseases.

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