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Review

The development of gene therapy for diseases of the lung

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Abstract. The development of a successful gene therapy has many stages, including preclinical testing in animal models and proof of principle clinical studies. A variety of diseases affect the lung, which are candidates for gene therapy; this review will mainly focus on the diseases that have attracted the most attention and have therefore yielded the most progress, namely lung cancer and the monogenic disorder cystic fibrosis. Knowledge gained from clinical studies could eventually be applied to more complex lung conditions such as acute respiratory distress syndrome and asthma. In addition, increased gene transfer efficiencies could be obtained by appropriate selection of the gene transfer vector and mode of delivery.

Key words. Gene therapy; lung disease; cystic fibrosis; emphysema; adenovirus; adeno-associated virus; DNA/liposome; promoter attenuation.

Introduction

The lung is an important target organ for gene therapy of many acute and chronic diseases, including cancer, asthma, cystic fibrosis, alpha-1-antitrypsin deficiency and respiratory distress syndrome, among others. Many studies have demonstrated the feasibility of lung gene transfer, and recent progress will be reviewed concentrating on research that has been validated in animal models or in the clinic. The majority of advances have been made in the treatment of lung cancer and cystic fibrosis, and these will be considered as representative of diseases, which require acute and chronic lung gene expression, respectively. The lung is a particularly attractive target organ due to relatively non-invasive accessibility through the airways and vasculature, and the availability of well-developed technologies for the delivery of aerosols. However, once in the lung, gene transfer vectors can encounter highly effective defences that have evolved to protect the airways from particles of all sizes, including allergens, viruses and bacteria. Thus, the topical delivery of a gene transfer agent (GTA) meets with several considerable extracellular barriers before cell binding and entry can take place. Once inside the cell, vectors must negotiate endosomal release and nuclear translocation, and avoid promoter attenuation in response to vector-induced immune and inflammatory responses. Correction of surrogate disease endpoints has been observed in proof-of-principle clinical studies, and as vector technology evolves, gene therapy is likely to become a reality for a number of lung diseases.

Gene transfer agents for the lung

A variety of viral and non-viral gene transfer vectors have been evaluated in the lung. Retroviruses, perhaps the most widely used of all gene transfer vectors, are capable of long-term gene expression following genomic integration, but have limited applicability because they fail to efficiently transduce the non-dividing, terminally differentiated cells that make up the bulk of the lung [1]. However, Lentiviruses, a subclass of retrovirus that can transduce

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terminally differentiated cells, have been developed as gene transfer vectors [2]. Adenovirus (Ad), a double-stranded DNA virus that has been extensively used as a non-integrating GTA in the lung, infects nonreplicating cells and shows tropism for airway cells [3, 4]. The application of Ad vectors for chronic disease has been limited by the host's immune responses generated after administration. First-generation adenoviral vectors were deleted for the early region (E1) genes to make the virus replication defective [5]. Third-generation Ad vectors are 'gutless' or 'helper-dependent', retaining only a small packaging signal and inverted terminal repeats in order to reduce the host immune response to viral gene products [6]. Adeno-associated virus (AAV), a non-pathogenic parvovirus, encodes only two proteins, Rep and Cap, both of which are deleted in the recombinant vector to reduce potential immune responses [7]. AAV vectors tested in the airways show potential for persistent expression whilst being maintained as an episome, or integrated into the genome [8]. Sendai Virus (SeV), also known as haemagglutinating virus of Japan, is a negative-strand RNA paramyxovirus that has a complex RNA-based cytoplasmic cycle [9]. It has the advantage of being naturally tropic for the airway epithelial cells and is therefore being evaluated in the lung. Many of these viral vectors are efficient at transducing lung cells under the right conditions; however they all generate inflammatory and immune responses to some degree.

To combat potential, viral vector toxicity, there has been an explosion of research into non-viral vectors, the simplest being naked plasmid DNA, where transgene expression is controlled only by the specific sequences contained in the plasmid [10]. Expression from naked DNA may be restricted by its vulnerability to degradation and low mobility in the cytoplasm [11]. The majority of non-viral vectors consist of plasmid DNA complexed with lipids or polycations, such as poly-L-lysine or polyethylenimine (PEI), to compact the large plasmid molecule and to protect it from degradation [12, 13]. Cationic lipids are amphipathic molecules with a positively charged headgroup capable of interacting with the negatively charged backbone of DNA and hydrophobic tail groups [14] and have been widely tested in the airways. Non-viral complexes can be further modified to include peptides with the potential for cell targeting and nuclear translocation [15].

Delivery to the lung

The lung is a complex organ, which, for the purposes of this review, will be divided into the conducting large and small airways (trachea, bronchi, bronchioles), and the parenchyma (gas-exchanging alveolar cells). The tracheobronchial tree extends from the trachea down through numerous divisions of airways lined with epithelium. In the larger airways, the epithelium is pseudostratified, consisting of ciliated and non-ciliated columnar cells, goblet cells, as well as rare neuroendocrine cells and a layer of basal cells [16]. The epithelium eventually transitions to a single layer of cells in the respiratory bronchioles, consisting of cuboidal ciliated cells, and columnar Clara cells that are the progenitors of the bronchiolar epithelium [17, 18], although the distribution of these cell types appears to be species specific [16]. In the parenchyma of the lung, the alveoli are lined with numerous large, flat, gas-exchanging, type I pneumocytes, along with cuboidal, surfactant-secreting, type II pneumocytes (see also fig. 1). The parenchyma forms a very large surface area (~100 m^2) up to 99 % of which is made up of type I cells [19]. This region is also highly vascularised, with capillary endothelial cells accounting for 30 % of total cells in the human lung. The relative number and distribution of cell types may vary in different animal models.

Vector delivery to the airways has taken one of three routes: direct injection, systemic delivery and topical delivery via instillation or aerosolisation. Direct injection into a tumour or pleural space has been the delivery strategy for a number of lung cancers. Systemic delivery via intravenous injection is an attractive option. The lung has two circulatory systems, the pulmonary vasculature, which facilitates gas exchange, and the bronchial vasculature, which supplies nutrients to the airways. However, the cell types available for gene transfer via these vasculatures will vary depending on the species. The majority of studies have assessed gene transfer in the mouse lung, where the pulmonary circulation supplies the majority of the airways [20]. Typically, intravenous delivery of a GTA results in high parenchymal transfection, consisting mainly of pulmonary endothelial cells, due to the large surface area of the pulmonary microvasculature being the first capillary bed encountered by the GTA following intravenous injection [21–27]. A few studies have also demonstrated transfection of alveolar epithelial cells (type I and II) by this route [25, 27–29].

There are very few reports of consistent transfection of the conducting airways following systemic delivery, which would require the GTA to escape from the capillaries and diffuse through adjacent tissue to transfect airway epithelial cells. However, it is possible that cell-specific, receptor-mediated uptake might enhance gene transfer efficiency following systemic delivery. For example, transfection using the polymeric immunoglobulin receptor, present on epithelial cells in the large airways, resulted in transgene expression in airway epithelial cells (17%) and some submucosal glands following systemic delivery in rats [30]. The distribution of transfected cells suggests that the GTA may have been delivered via the bronchial circulation. Airway epithelial cells can be transfected by small oligonucleotides that exit the bronchial circulation; transfection was mainly cytoplasmic, but a punctate and

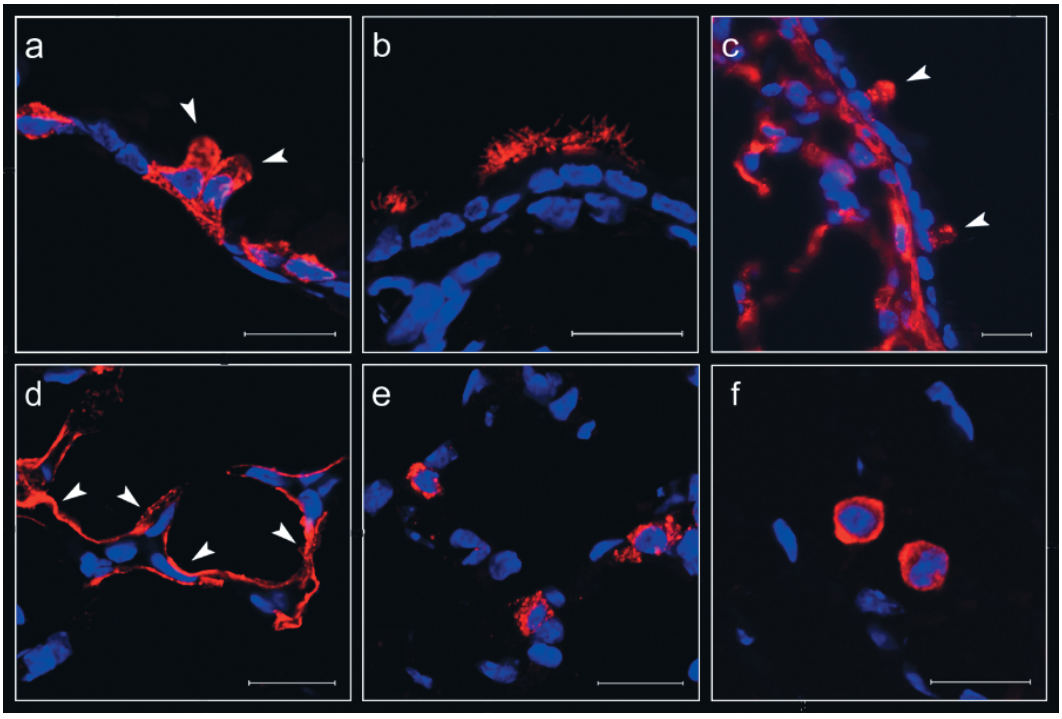

Figure 1. Identification of major cell types in mouse lung. Murine lung cells identified using immunohistochemical analysis with cell-type-specific antibodies labelled red with AlexaFluor 546 (Molecular Probes, Leiden, Netherlands) and visualised using confocal microscopy. (*A*) Clara cells (arrow heads) labelled with anti-human urine-protein 1 antibody (Dako, Ely, UK), (*B*) ciliated cells labelled with anti-β-tubulin IV antibody (BioGenex, San Ramon, CA, USA), (*C*) goblet cells (arrows) labelled with anti-MUC5 AC antibody (Neomarkers, Fremont, CA, USA), (*D*) type I pneumocytes (arrows) labelled with anti-T1alpha antibody [182], (*E*) Type II pneumocytes, labelled with anti-surfactant protein B antibody (Chemicon, Temecula, CA, USA), (*F*) alveolar macrophages labelled with anti- F4/80 antibody (Serotec, Oxford, UK). Nuclei are stained blue with the nuclear stain TOTO-3 (Molecular Probes). Bar represents 20 μm.

diffuse nuclear localised signal was also observed [31]. In one study, bronchial epithelium and tracheal submucosal glands were successfully transfected [29], although in many other studies this was not the case, suggesting that this is rare and may be dependent on the GTA used. An understanding of the structure and charge characteristics of the GTA may be particularly important. Positively charged GTAs may become neutralised by plasma serum proteins, form aggregates and become trapped in the pulmonary microvasculature, whereas neutral and negatively charged particles are maintained in the circulation longer and transfect the lung only poorly, favouring gene delivery to the liver [26, 32, 33]. Viral vectors will require cell receptors accessible via this route [34]. Thus selection of the appropriate GTA for the required cell type is crucial. In addition, careful interpretation of these studies in the light of differences between animal models and humans will be required for translation of these results into the clinic.

In many animal studies, topical delivery to the lung is achieved by instillation of a bolus of fluid, either directly via intratracheal injection [25] or via intranasal sniffing [35]. Whereas systemic delivery leads to transfection of pulmonary endothelial cells and some alveolar cells, topical delivery leads to transfection of the airway cells [25]. One advantage of topical over systemic delivery is that gene transfer is targeted to the lung, thus limiting gene transfer to other organs (including the gonads), which may minimise unwanted toxicity. For example, systemic delivery of PEI, a polycation capable of binding and compacting DNA to protect it from degradation [36, 37], generated a substantial cytokine response in vivo and high mortality [38–40]. However, when similar formulations were aerosolised to the lung, toxicity was minimal [41, 42]. Aerosolisation of a liquid formulation into inspirable droplets distributes the GTA throughout the lung [43] and is the likely delivery option in patients. Whereas aerosol delivery is well advanced in the clinic, this can be difficult to model in small rodents, due to the smaller airway diameter, low tidal volumes and consequent poor deposition rates [44]. Larger animal models with lung size and architecture similar to humans have been used, including non-human primates [45], pigs [46] and sheep [47]. Aerosol delivery to large animal models may require substantial reformulation efforts involving significant increases in production and cost [48]. Nevertheless, non-invasive access to the lung surface via well-tested aerosol delivery technologies will be required for routine and repeated airway delivery to patients.

Extracellular barriers to lung gene transfer

Once inside the lung, GTAs meet both physical and immunological barriers to gene transfer (reviewed [49, 50]). Normal mucociliary clearance mechanisms responsible for eliminating small particles from the lung may also eliminate GTAs. Studies show that the glycocalyx on the apical surface of epithelial cells represents a significant barrier to viral vectors; addition of neuraminidase to remove the sialic acid component from polarized epithelial cells leads to enhanced gene transfer [51]. The surface of the epithelial cell itself also appears resistant to vector binding and uptake. Several studies show that non-viral gene transfer to polarised airway epithelial cells is reduced as cells become more differentiated in culture [52–54]. The reason for this is unknown, but correlates with increasing levels of polarisation, differentiation and tight junction formation. It is also possible that low levels of internalisation and endocytosis may limit GTA uptake at the apical surface [55]. These findings underline the importance of evaluating gene transfer efficiency in well-differentiated airway cells rather than in simple, immortalised cell culture systems. In addition, the lung may have relatively few viral receptors located on its apical surface, possibly to minimise viral infection. Ad vector transduction of airway epithelial cells appears relatively inefficient from the apical surface [55] and may occur only after achieving access to receptors on the basolateral surface following lung injury. Enhanced viral gene transfer can be observed following disruption of epithelial tight junctions by the addition of calcium chelators [56, 57], or compounds affecting paracellular permeability such as sodium caprate [58] and polidocanol [59].

Further barriers to lung gene transfer include the host's innate immune defences. Immune cells such as alveolar macrophages can ingest GTAs by a mechanism probably involving phagocytosis [60], and when macrophages were eliminated from the mouse lung there was a 96% increase in reporter gene expression compared with control animals [61]. Moreover, lung delivery of both viral and non-viral vectors can result in nonspecific inflammation, which can lead to inhibition of transgene expression [62]. Both the cellular and humoral immune responses can be activated in the presence of foreign proteins, thereby restricting vector re-administration, which is a serious obstacle to be overcome for the treatment of chronic diseases [49]. However, stimulation of the inflammatory/immune cascade may represent an advantage for diseases such as cancer. Physical and immune barriers in the lung are summarised in figure 2.

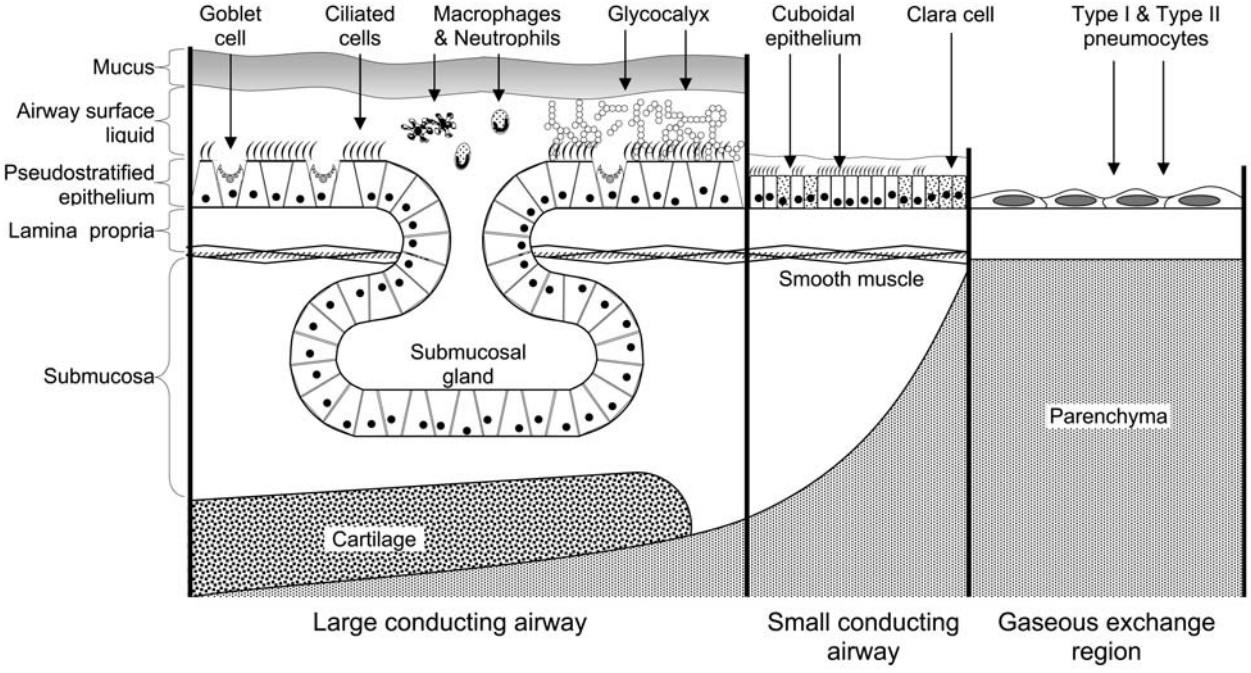

Figure 2. Barriers to successful lung gene transfer**.** The surface epithelium of the large conducting airways (bronchi) is pseudostratified and is covered by a thick layer of mucus and airway surface liquid (ASL). ASL comprises of antimicrobial proteins, lactoferrin, lysozyme and defensins in addition to water and also contains the epithelial glycocalyx. Neutrophils and alveolar macrophages may also be resident in this layer. The bronchi contain cartilage, smooth muscle and a large submucosal region which decreases as the airways become smaller. The epithelium of the small conducting airways (bronchioles) has a simpler, columnar/cuboidal organisation containing mainly ciliated epithelial cells and Clara cells. The gaseous exchange region is composed of the respiratory bronchioles with the epithelium interrupted by the alveolar ducts, which contain the alveolar spaces.

Gene therapy for acute and aquired lung disease

For treatment of lung cancer, acute transplant rejection and lung injury as a result of radiotherapy-induced damage, and so on, gene transfer vectors capable of rapid onset, high-level, transient gene expression are required. Induction of transgene expression from Ad vectors occurs rapidly in the lung, peaking 3–4 days following administration, with expression falling to baseline levels within a few weeks [63]. Ad binds to the high-affinity Coxsackie and adenoviral receptor (CAR) and the $\alpha_v\beta_5$ integrin [64] located on the basolateral surface of the lower airways [65]. Thus, damage in the lung may potentially expose the basolateral surface receptors and facilitate localised Ad transduction. In addition, in mouse models of acute lung injury induced by bleomycin or lipopolysaccharide, Ad vector administration did not result in additional lung inflammation [66, 67].

Lung injury

Bronchiolitis obliterans, a form of chronic graft rejection, is a major obstacle to improving long-term survival for lung transplantation. An important predictor is the severity of acute lung injury during the early stages of transplantation, leading to cell-mediated inflammation [68]. Whereas gene therapy for bronchiolitis obliterans may require long-term gene expression, perhaps for years, gene therapy for reducing inflammation in the very early stages may also be therapeutic. Both Ad vectors and DNA/liposome complexes have been tested in rat lung transplantation models, demonstrating successful reporter gene expression following transplantation [69–71]. Importantly, the timing and route of delivery appear crucial to success. Several transgenes have been delivered with the aim of reducing acute rejection, including anti-inflammatory interleukin (IL-10) [72], nitric oxide synthase [73], transforming growth factor beta-1 [74], Fas ligand [75] and CTLA4Ig [76]. Results are encouraging, but improved gene transfer efficiency is required for therapeutic benefit in the grafted lung. The possibility of radioprotective gene therapy to treat acute tissue damage following irradiation for cancer treatment has also been investigated. Adenoviral and DNA/liposome-mediated delivery of the transgene encoding the antioxidant manganese superoxide dismutase has been tested in the rodent lung [77]. Intratracheal delivery of DNA/liposomes to the mouse lung prior to irradiation resulted in transient transgene expression in epithelial and parenchymal cell types [78]. In all these applications, the judicious selection of GTAs with reduced inflammation and the appropriate transgene expression profile should now be evaluated.

Lung cancer

Lung cancer can result from the accumulation of mutations leading to cellular transformation and the development of invasive metastatic disease [79]. Malignancies that are resistant to both chemotherapy and radiotherapy are targets for gene therapy, with the specific aim of destroying the tumour cells in situ. One approach is the delivery of tumour suppressor genes such as p53 [80] and the K-*ras* oncogene, where expression can be downregulated by delivery of antisense [81], or hammerhead ribozyme transgenes [82]. It is anticipated that the enormous interest in the use of RNA interference (RNAi)-mediated strategies to downregulate gene expression [83] will also have a significant impact in this field. Direct injection of retroviral vectors expressing p53 into nine patients with non-small-cell lung cancers showed tumour regression in three patients and tumour stabilisation in a further three patients despite low levels of gene transfer [84]. Similar results were obtained using Ad vectors [85, 86]. Novel Ad vectors capable of selectively replicating in p53-mutant cells have been intravenously infused into patients with advanced lung metastases, resulting in intratumoral viral replication in three out of four patients [87]. A similar study showed that monthly delivery of Ad expressing p53 was well tolerated with only minor side effects [88]. The cationic polymer PEI was also used to repeatedly deliver p53 to rodent lungs by aerosol, resulting in widespread transgene expression throughout the lung and a significant reduction in the lung tumour burden in murine models [89, 90]. The precise mechanism of action is unclear but could involve alterations in the expression of anti-angiogenic factors [91]. Using gene transfer to directly inhibit tumour angiogenesis has also been explored [92]. Systemic delivery of recombinant Ad expressing the potent angiogenesis inhibitor endostatin resulted in reduced growth rates of breast and lung carcinoma and prevented formation of pulmonary micrometastases in animal models [93].

Pro-drug or suicide gene therapy is an approach that has been used to transfer genes that sensitise tumour cells to otherwise nontoxic drugs [94]. The transfer of herpes simplex virus thymidine kinase (HSVTK) DNA to tumour cells has been achieved with both viral and non-viral vectors [95, 96]. First-generation Ad vectors expressing HSVTK were used in a phase I dose escalation study to treat patients with mesothelioma [97,98]. Intratumoral gene transfer was achieved in more than half the treated patients, with partial tumour regression in some cases, although strong anti-adenoviral immune responses were also observed that could not be reduced by simultaneous immunosuppression at the time of vector delivery [99]. Although the 'bystander effect' [100] is helpful to enhance efficacy where gene transfer is limiting, increased gene transfer efficiencies will be required to reduce tumour burden to the degree required for clinical improvement.

Immunogenetic therapy involves the recruitment of the host's immune system to destroy tumour cells by delivery of transgenes such as cytokines and co-stimulatory molecules [101]. Local expression of factors affecting the immune system may stimulate cascade responses, thereby amplifying potential anti-tumour effects. IL-12 was expressed in the lungs of a nude mouse model of osteosarcoma lung metastasis following intranasal administration of PEI complexes twice weekly for 4 weeks, resulting in a reduction in the number and size of lung metastases [102]. Replication-restricted vaccinia virus expressing IL-2 was injected into the chest wall lesions of patients with advanced malignant mesothelioma; evidence of gene expression was detected, but no tumour regression was observed [103]. Currently the most potent transgenes are unknown, but a nonspecific approach may be sufficient. Systemic delivery of DNA/liposomes to mice triggered release of high levels of IL-12 and interferon (IFN)-γ, resulting in rejection of pulmonary metastases [104]. Both innate and adaptive immune responses appear to be elicited by the DNA/liposomes, irrespective of the transgene expressed from the plasmid. Although the unmethylated CpG motifs found in bacterial DNA [105] were shown to be crucial, delivery of plasmid DNA alone was not sufficient; DNA/lipid complexes were required to generate the anti-tumour responses [106].

The precise molecular mechanisms of tumour regression using gene therapy strategies are unclear and may require the bystander effect, or induction of secondary immune responses [107]. A significant issue for lung cancer gene therapy is to understand the local cytokine pathways in the context of the immune system in order to amplify (and control) the response. Moreover, with only minimal evidence for gene transfer to tumour cells in vivo, improving gene transfer efficiency to the required cell types is an important goal.

Gene therapy for chronic lung disease

For many chronic lung conditions such as asthma, cystic fibrosis and emphysema, persistent transgene expression in slowly dividing or terminally differentiated lung cells will be needed. Therefore, the ability to repeatedly administer a specific gene transfer vector becomes a pressing priority for these diseases. Pulmonary emphysema due to loss of elastin from the lung parenchyma can be caused by a deficiency in α1-antitrypsin (AAT), an abundant serum protein produced predominantly in the liver that functions as a major anti-protease, counteracting the effects of neutrophil elastase and other pro-inflammatory molecules released at sites of inflammation [108]. Current protein therapy requiring weekly intravenous administration is costly and may expose recipients to the risk of viral transmission from human serum [109]; gene therapy is being evaluated as an alternative approach. Early studies in cotton rats using first-generation Ad vectors resulted in detection of AAT in bronchoalveolar fluid for only 1 week post-administration [110]. Early problems experienced with transient gene expression and vector-induced inflammatory responses could now be addressed with the use of helper-dependent Ad and suitable promoter sequences, but the absence of the required viral receptors still limits the use of Ad in the human lung for chronic disease. Cationic liposomes have also been used to express human AAT in the rabbit lung following aerosolisation [111]. In a non-blinded study, the human AAT gene complexed with cationic lipid (DOTMA/DOPE) was delivered to the nasal epithelium of patients with AAT deficiency and resulted in nasal lavage samples which were transiently positive for transgene messenger RNA (mRNA) [112, 113]. Recombinant AAV vectors are being evaluated for more persistent expression of therapeutic serum levels of human AAT in murine and non-human primate models following intramuscular injection [114, 115]. Secretion of the AAT might also benefit the liver aspects of the disease.

The chronic lung disease that has received most attention in the field of gene therapy is cystic fibrosis (CF). This monogenic disorder is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, leading to abnormal secretions, recurrent infection, inflammation and premature death [116]. Although a multi-organ disease, the majority of morbidity and mortality is associated with the lung, thus CFTR replacement lung gene therapy has been intensively investigated. CFTR encodes a cyclic AMP (cAMP)-regulated chloride channel, and it is hypothesised that CFTR gene transfer to the epithelial cells of the small airways, where the disease begins, is likely to be required for therapeutic benefit [117–119]. In addition to the usual barriers to lung gene transfer, the CF lung suffers from abnormally thick mucus secretions, an inflammatory milieu and bacterial colonisation. CF sputum and bronchoalveolar lavage fluid have been shown to block the transport of small particles [120] and reduce viral and non-viral gene transfer [121–123]. However, the damaged CF lung may also result in higher levels of epithelial turnover and remodelling, leading to increased opportunities for gene transfer with certain vectors.

Adenovirus for cystic fibrosis gene therapy

Adenovirus was the first viral vector tested for CF lung gene therapy, with early studies in rodents suggesting that Ad may be an efficient GTA for lung gene therapy (see also fig. 3). Ad vectors were aerosolised to the lungs of cotton rats and mice, resulting in reporter gene expression in up to 10–30 % of the surface epithelium in the airways [124]. When Ad-CFTR was delivered to the airway ep-

ithelium of CF transgenic mice, CFTR-dependent chloride conductance was partially corrected for up to 15 days post-administration, while *CFTR* mRNA was detected for up to 70 days [125]. These studies led to a number of single and repeat administration phase I trials in humans, some of which showed functional changes in the nasal epithelium consistent with the expression of CFTR [126, 127]. Other trials have highlighted problems with anti-adenovirus antibody responses and inflammation [128]. The high efficiency of gene transfer observed in mouse models was not seen in CF clinical trials. In the cells of the nasal epithelium, upper airways and trachea, the Ad receptor CAR is situated on the apical surface of cells, but in the lower airways it is restricted to the basolateral surface [129]. The paucity and distribution of CAR in the human airways is the likely cause of inefficient gene transfer in the absence of damage or disruption of tight junctions [130]. The correction of CF lung pathology will likely require long-term CFTR expression, and Ad readministration shows limited success due to inflammation, neutralising antibodies and cytotoxic T lymphocyte (CTL) responses induced by the initial delivery [131]. In a study delivering three doses of an Ad-CFTR vector, 3 months apart, to the lobar bronchus of patients with CF, the levels of vector derived mRNA were reduced after the second dose and undetectable by the third dose [132]. The localisation of CAR in airway cells and the loss of efficacy upon repeated administration have limited the applicability of Ad vectors for CF gene therapy.

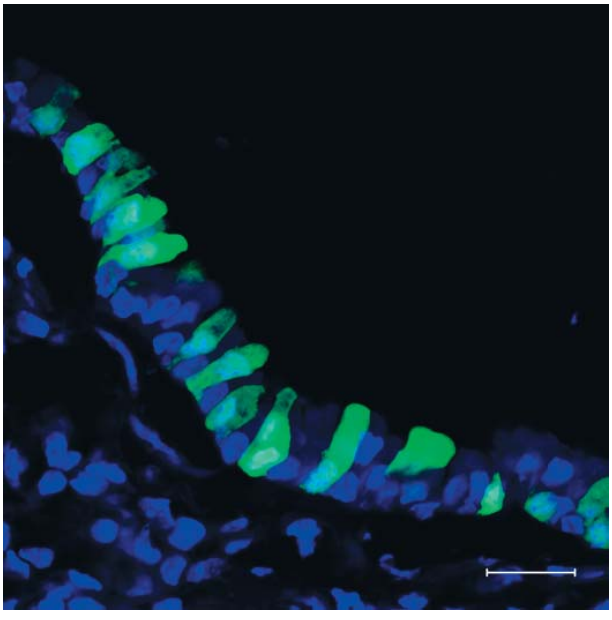

Figure 3. Efficient adenoviral gene transfer to the murine airways. Green fluorescent protein (GFP) transgene expression in Clara cells lining the mouse bronchus, following intratracheal administration of 1×10^7 pfu of the adenoviral vector AdMCMV-GFP (kind gift of Dr F. Graham, McMaster University, Hamilton, ON, Canada). Bar represents 20 μm.

Adeno-associated virus for CF gene therapy

The testing of AAV in the clinic has been facilitated by improvements in the manufacture of large quantities of AAV vectors [133] and in strategies to overcome DNA packaging constraints [134, 135], using synthetic promoters and minigenes [136, 137]. Delivery of AAV (serotype 2) vectors has been evaluated in the rabbit lung, where AAV offers long-term transgene expression, possibly for the lifetime of the transduced lung cells [138]. Similarly, after delivery of AAV2-CFTR to rhesus monkey lungs, vector-specific DNA was detected in all animals, and mRNA was detected at 180 days post-administration [139]. No adverse side effects were noted, and there was no evidence of inflammation in the lungs. However, gene transfer with AAV2 was mainly found in type II alveolar cells [140], or areas damaged following delivery [141], rather than the airway epithelial cells required for CF gene therapy. Several re-administration studies have been performed, but conflicting results obtained. In rabbits, anti-AAV neutralising antibodies were detected in the blood of animals after primary administration, and no positive cells could be detected in the lungs [141]. However, in a separate study, green fluorescent protein (GFP) expression could be detected in the rabbit lung following two subsequent administrations of AAV and despite the presence of circulating neutralising antibodies [142]. In addition, immunomodulation to suppress the production of neutralising antibodies has allowed the successful re-administration of AAV in mice [143]. AAV2 vectors expressing CFTR have been tested in CF patients using the respiratory epithelium lining the maxilliary sinus as a surrogate for the lung [144–146]. A dose-escalation, phase I study demonstrated the safe aerosolisation of up to 10^{13} DNase-resistant particles of AAV2 to the lungs of CF patients [147], and further clinical studies are underway to assess efficacy. Membrane-associated heparan sulphate identified as a receptor for AAV2 [148] is localised on the basolateral surface of airway epithelial cells [149], and therefore is not easily accessible via topical administration unless tight junctions are disrupted to allow access [150]. This may explain the preference of AAV2 to transduce parenchymal cells [151] and may limit its application for CF. However, alternative AAV serotypes with tropism for the airway epithelia are now being investigated [151–154].

AAV vectors may be useful for other diseases where parenchymal gene transfer is required or tolerated, such as the production of therapeutic proteins for diseases not necessarily associated with the lung itself. The lung has a large surface area and good vascularisation that could allow it to function as a 'protein factory' resulting in local and systemic release of proteins into the blood. In mice, recombinant AAV vectors have been used to deliver erythropoietin and factor IX into the systemic circulation following lung gene transfer [155]. Following a single in-

tranasal administration of AAV2 vector pseudotyped with capsid from AAV serotype 5, protein expression could be detected for up to 150 days. This study also demonstrated that the recombinant AAV2/5 vector could be effectively readministered to the lung 5 months after the first delivery, evidence that AAV might be a suitable candidate vector for persistent, repeatedly administered gene transfer for chronic lung diseases.

Other viral vectors for CF gene therapy

Lentivirus expressing CFTR was delivered to the nasal epithelium of CF transgenic mice and was shown to partially correct the chloride ion transport defect in the nose for up to 110 days [156]. However, in order to achieve transduction of the ciliated epithelial cells, tight junctions in the epithelium were disrupted to provide vector access to the basolateral surface, a strategy which may not be acceptable in patients [157]. An alternative approach is to use vector pseudotyping, whereby a ligand specific for a cell-surface receptor is added to the viral vector to alter tissue tropism. A human immunodeficiency virus (HIV)-based vector has been pseudotyped with an *Ebola* virus envelope (Ebo-HIV), a human pathogen with tropism for the airways [158, 159]. Following instillation of Ebo-HIV into mouse lungs, widespread reporter gene expression was observed for up to 63 days in the alveoli, small and large airways and in the submucosal glands. Efficient gene transfer was also observed in explants of human trachea [159]. Although repeat administration is unlikely to be efficient due to neutralising antibodies and cell-mediated immunity, the potential for transducing and integrating into lung progenitor cells cannot be ignored [156]. Recently, SeV has emerged as a gene therapy vector with excellent targeting of the airway epithelia [160]. The presence of the receptor on the surface of the conducting airway epithelia means that there is no need to access the basolateral surface of the airways through pseudotyping or opening tight junctions. Replication defective SeV vectors are under evaluation, but once again it is likely that the virus will elicit an immune response, which is expected to greatly inhibit re-administration of this vector.

DNA/liposome complexes for CF gene therapy

Whereas anti-viral immune responses make repeat delivery problematic for viral vectors, non-viral vectors may be sufficiently less immunogenic in the lung to allow repeat administration. Plasmid DNA complexed with cationic lipids (DNA/liposomes) has been tested both in animals and in the clinic. Correction of CFTR ion transport defects in CF transgenic animals [161, 162] supported clinical studies to test the safety and efficacy of a variety of cationic liposomes in the nasal epithelium of CF patients [65, 163–167]. Three double-blind studies demonstrated proof of principle using DC-Chol:DOPE [163, 164] or DOTAP [165]. In addition to detecting vector-specific DNA and mRNA, all three studies showed partial correction of the CFTR ion transport defect in the nose, in one case extending out to 15 days post-administration [164]. Importantly, one study evaluated the effects of three doses of DNA/liposomes to the nose at 1 month intervals, and saw no evidence of inflammatory or immune responses [166]. In addition, efficacy after the third dose was comparable to earlier doses, suggesting that unlike repeated delivery of adenovirus to the nasal epithelium where efficacy was reduced upon re-administration [168], repeated delivery of cationic liposomes was effective, at least in the nose.

Improvements in the design of cationic lipids have resulted in more efficient non-viral formulations [48]. When complexed, Genzyme lipid 67 (GL-67) plasmid DNA generated reporter gene expression in the mouse lung up to 100-fold greater than other liposome formulations [169], although expression was transient, lasting only a few days after administration. Two studies have evaluated these complexes in the CF lung [170, 171]; delivery of DNA/GL-67 liposomes by aerosol resulted in partial, but significant correction of chloride ion transport in the lungs of CF patients [170]. However, both studies reported side-effects consisting of a transient fever in patients receiving the active formulation accompanied by an elevation in IL-6 levels. The side-effects were thought to be mainly due to the inflammatory response caused by the recognition of bacterial CpG motifs contained within plasmid DNA, which appears to be increased when the plasmid DNA is complexed with liposomes [172, 173]. Whereas this type of response, including the production of pro-inflammatory cytokines IL-4, IL-12, IL-1β, IFN-γ and tumour necrosis factor alpha (TNF-α) [173] may be tolerated and possibly beneficial for cancer gene therapy, it is likely to be disadvantageous for long-term treatment of chronic disease. However, this problem may be specific to liposome-mediated gene transfer; some other non-viral vectors do not appear to generate such a vigorous CpG dependent response [42, 174].

The development of novel non-viral vectors

A variety of novel synthetic vectors are emerging for in vivo testing, including DNA complexed with cationic polymers such as PEI and poly-L-lysine [13]. In addition, the incorporation of targeting peptides into DNA/liposome complexes may increase cell specificity and uptake, thereby increasing overall gene transfer efficiency. Plasmin^{R} is a complex of plasmid and PEG-substituted polylysine with the potential advantage of forming small nanoparticles (<25 nm diameter), which may be small enough to pass through the nuclear pore, thereby enhanc-

ing gene expression once inside the cell [175]. This formulation has been tested in murine airways [176] and is currently being evaluated in the nasal epithelium of CF patients. Similar complexes with the addition of the SecR (serpin enzyme complex receptor)-targeting peptide showed correction of the chloride ion transport defect in the nasal epithelium of CF transgenic mice [177]. Control complexes lacking the targeting ligand did not lead to gene expression or physiological correction, emphasising the importance of receptor targeting in this system. The costs involved in synthesising large quantities of peptide for testing in large animal models may be prohibitive, and the development of stable formulations of lipid-peptide-DNA combinations for aerosolisation, is not trivial [46]. Synthetic peptides specific for airway epithelia have also been used; a synthetic peptide combining a poly-lysine DNA binding domain and an $\alpha_5\beta_1$ integrin binding domain into DNA/liposomes can transfect the airway epithelial cells of pig lung when delivered by bronchoscopy [46]. The inclusion of such peptides into non-viral formulations may have wide applicability; however, it also begins to erode some of the potential advantages of a non-viral vector. If repeated administration is required for treatment of chronic disease, then anti-peptide immune responses might result if the sequence is not completely-human derived.

A common component shared by non-viral vectors is plasmid DNA, and modification to enhance gene expression and reduce toxicity should not be neglected. Reduction of immune-stimulatory CpG motifs in the plasmid sequences can reduce inflammatory side-effects following delivery of DNA/liposomes [178, 179]. This is also important because the elevation of inflammatory cytokines can impact on transgene expression. Strong viral promoter sequences, such as the cytomegalovirus immediate/early promoter, have been used for nonviral gene transfer studies to achieve high expression levels in vivo but are transcriptionally regulated by cellular factors sensitive to inflammatory cytokines [180]. Transgene expression from such promoters can become attenuated in response to vector-induced inflammation in vivo, leading to transient gene expression despite the persistence of vector in the cell [62]. Thus, there may be a complex, bidirectional interaction between vector-induced inflammation and transcription factors in the target cell which impacts on transgene expression (reviewed in [181]). Endogenous promoters appear to be less affected [62]; in the mouse lung, the use of the human polyubiquitin C promoter in naked plasmid DNA extended expression from 1 week to several months, possibly for the lifetime of the transfected lung cells [35]. The choice of promoter can also improve cell specificity. The cytokeratin 18 promoter has been shown to favour epithelial cell expression in the mouse lung [29]. Although plasmids incorporating such features have not yet been tested in the human lung, such improvements in plasmid design could improve gene transfer efficiencies in the clinic.

Conclusions

Several viral and non-viral GTAs have been evaluated for a range of conditions in animal models and in the clinic. Both physical and immunological barriers exist, and these must be tackled. Although encouraging progress has been made, particularly for lung cancer and CF, all these studies are plagued by low levels of gene transfer. From this review, it has emerged that a single vector is highly unlikely to be optimal for all lung gene therapy applications. To improve gene transfer efficiency, the appropriate vector must be selected for transfection of the required cell type. Ultimately, all disease targets will benefit from an improved understanding of the biology of vector delivery, uptake and expression. Improvements in vector design will also reap rewards in the clinic. Research into vector design and targeting needs to proceed in parallel with early clinical studies, where proof of concept and identification of barriers to clinical gene transfer can inform the next phase of basic research. In the absence of the perfect animal disease model, this iterative process has become the norm for gene therapy research and should ultimately lead to success.

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