

Gene therapy for respiratory diseases: potential applications and difficulties

The use of genetic materials for treatment is a relatively simple concept which, if successful, could revolutionise the management of both inherited and acquired disease. The aim is to modify production of a specific protein either through upregulation by introducing the DNA of interest, or downregulation through the use of antisense oligonucleotides. Such modification could be for long term correction of an inherited genetic defect such as cystic fibrosis or α_1 -antitrypsin deficiency, on the one hand, or an acquired defect such as cancer on the other. Alternatively, short term modification of protein production could be used to alter inflammation in conditions such as asthma and oxidant-mediated lung injury. Although the concepts are simple, the technology is complex and poorly understood, and it will be some time before gene therapy is routinely available in clinical practice. This editorial outlines the technology of gene transfer and reviews progress towards therapy for the three lung conditions with approved gene therapy protocols, namely cystic fibrosis, α_1 -antitrypsin deficiency, and lung cancer.

Gene transfer techniques

Gene transfer requires two principal components – the cDNA of interest linked to an appropriate promoter, and a gene transfer agent to facilitate entry into the cell. Most current studies use “ubiquitous” promoters such as SV40, CMV and RSV which are derived from eukaryotic viruses and, in theory, are active in any nucleated cell. Lung specific promoters such as SP-C (surfactant protein C) and CC 10 (Clara cell) are also being investigated.¹ There has, to date, been relatively little work done to define the relative advantages of the different promoters. It is likely, however, that cell specificity will prove to be valuable as it will limit gene expression to a desired cell population and may also be easier to control.

The two main approaches being investigated with regard to gene transfer agents are the use of viruses and liposomes. Each has certain advantages and problems and future systems may combine the best features of both.

Retroviruses can produce efficient gene transfer, including the integration of the DNA into the host genome. These viruses are well suited to *ex vivo* transfection of cells which are removed from the body and then replaced in a modified form as, for example, for hepatocytes and production of α_1 -antitrypsin considered below. They are, however, unsuitable for topical lung delivery since they only transfect dividing cells and not the terminally differentiated airway epithelium. Furthermore, *in vivo* use may carry the risks of germ line transfection and the induction of other unwanted changes by virtue of their random integration into the host genome.

Adenoviruses have the advantage of being trophic for the respiratory epithelium and should, in theory, produce relatively efficient gene transfer to the airways. However, two principal concerns about their use centre around safety and efficacy on repeated administration. Portions of the virus's own genome are deleted, partly to provide space for the gene of interest to be inserted and partly to produce a replication incompetent virus. The principal problem relates to the viral coat proteins which are immunogenic and which result in the production of inflammation through cytotoxic T lymphocytes or neutralising antibodies re-

ducing the efficiency and duration of repeated applications.² A second theoretical risk is recombination with a wild type virus which might restore the ability to replicate and cause infection. Increasingly extensive deletions of the viral genome are therefore being investigated to reduce immunogenicity and replication potential.³

Liposomes are lipid vesicles which form spontaneously when certain lipid preparations are mixed in polar solutions. Cationic liposomes form complexes with DNA (which has a net negative charge) and have been used for some years for *in vitro* gene transfer. Liposomes are relatively inefficient at gene transfer compared with viral vectors but have some major advantages. They have low toxicity and are unlikely to provoke inflammation or to initiate an immune reaction on repeated administration. Liposomes have complex physicochemical properties which are only poorly understood and which probably underlie their variable performance in gene transfer experiments.⁴

Administration of gene transfer systems

Nebulisation is likely to be the most acceptable delivery system for routine repeated application to the lower airways in man. It has the advantages of widespread deposition which can, to a limited extent, be controlled by varying droplet size. Contact time may also be enhanced by prolonged nebulisation, important in the light of mucociliary clearance within the airways. A theoretical disadvantage is the considerable mechanical stress applied to the liquid during the process of nebulisation. This might damage the gene transfer agent and, in particular, might alter the structure of a liposome-DNA complex. The presence of mucus and airway surface liquid further complicates predictions of whether efficient delivery can be achieved, particularly since purulent secretions contain DNases. The mucus layer may impair access to the cell surface, whilst the aqueous layer may dilute and alter the composition of any topically applied liquid. A recent report of pulmonary epithelial gene transfer following intravenous injection of a liposome-reporter gene complex is tantalising⁵ and, if repeatable, suggests the possibility of other gene transfer strategies. One potential disadvantage is widespread gene transfer to other organs, but organ or cell specific promoters or targeted gene transfer may help to overcome this difficulty.

Gene therapy for cystic fibrosis

Gene therapy for cystic fibrosis has become a possibility since the isolation of the gene in 1989.⁶ The protein coded for by this gene, cystic fibrosis transmembrane conductance regulator (CFTR), functions as a cAMP-regulated chloride channel on the mucosal surface of the airway epithelium.⁷ Initial studies demonstrated that *CFTR* gene transfer into cystic fibrosis cells *in vitro* could correct this chloride defect.^{8,9} Using both adenovirus¹⁰ and liposome¹¹ mediated gene transfer it was subsequently demonstrated that the *CFTR* gene could be expressed in the airways of normal mice *in vivo*. Instillation of the *CFTR* gene into the lungs was followed by appearance of mRNA at day 1, with expression sustained for up to four weeks. Transgenic cystic fibrosis mice have also been used to assess *CFTR* gene transfer *in vivo*. Thus, both instillation¹² and

nebulisation⁴ of *CFTR*-liposome complexes into the lungs of these animals has been shown to correct the chloride defect. However, the relatively large amounts of DNA used and the variability of correction suggest that inefficient gene transfer may be a problem with liposome-based systems. A number of studies using non-human primates have also reported *CFTR* expression following adenoviral-mediated gene transfer.^{13,14}

With respect to safety it is clear that, as discussed above, airway inflammation develops with increasing adenoviral titre administered. To date, liposome administration has not demonstrated any safety problems. Whether over-expression of *CFTR* is detrimental has been studied in a number of ways. Transgenic mice expressing the human *CFTR* gene in the distal airways and alveolar cells showed no adverse effects in terms of lung weight, morphology, or somatic growth.¹⁵ However, in vitro studies have shown a correlation between high *CFTR* expression and growth arrest of an epithelial cell line, whilst injection of rabbit embryos with *CFTR* cDNA caused the majority of the male offspring to be stillborn.¹⁶ Germ line insertion of the *CFTR* cDNA administered to the airways is more a theoretical than a real risk. *CFTR* is probably expressed in the gonads of non-cystic fibrosis subjects^{17,18} and males with cystic fibrosis are infertile. Furthermore, no germ line transfer was observed in primates following adenoviral-mediated reporter gene transfer to the lungs.¹⁴ These studies cannot, of course, exclude the possibility of such an event occurring very rarely.

Early data are beginning to appear from human studies. Zabner *et al*¹⁹ studied adenovirus-mediated *CFTR* cDNA gene transfer to the nose of three volunteers with cystic fibrosis. A degree of localised inflammation around the site of application was seen, probably related to the method of delivery. *CFTR* mRNA could be demonstrated in two of the subjects, whilst evidence for correction of the chloride abnormality was suggested. These changes lasted up to 10 days after the single application, although the study was not designed to assess duration of expression. Crystal *et al* have studied *CFTR* expression in the lungs of four patients following adenoviral-mediated gene transfer by direct instillation.²⁰ *CFTR* was shown in the cells taken from one of the patients, but mRNA could not be demonstrated. Assessment of correction of the chloride defect in the lungs was not attempted. One patient developed an episode of fever, hypotension and evidence of lung infiltrates probably related to the high viral titre administered. These changes reverted to normal over a 28 day period. We have recently completed a double blind placebo controlled trial of liposome-mediated *CFTR* cDNA gene transfer to the nasal epithelium in 15 delta 508 homozygous cystic fibrosis subjects (nine *CFTR* cDNA, six placebo).²¹ No safety problems were encountered either in the routine clinical assessment or by a blinded, semiquantitative analysis of nasal biopsies. Both plasmid DNA and *CFTR* mRNA were detected from the nasal biopsies in five of the eight treated patients. A significant 20% increase towards normal values was seen in the chloride measurements, with values reverting to cystic fibrosis values by approximately seven days. A number of other studies are underway and, whilst these early changes are encouraging, efficiency of gene transfer is likely to need improvement.

Gene therapy for α_1 -antitrypsin (AAT) deficiency

Although gene therapy approaches for this condition began at the same time as those for cystic fibrosis, progress has been slower. Since α_1 -antitrypsin is a circulating protein normally synthesised in the liver, it is not clear whether the gene should be targeted at the liver to mimic the normal

situation or at the lungs in order to achieve only local antiprotease protection. Furthermore, in comparison with cystic fibrosis it is likely that high levels of protein production will be required. These factors, as well as the uncertain progression of pulmonary disease even in the absence of a smoking history, make gene therapy for α_1 -antitrypsin deficiency a more difficult prospect than for cystic fibrosis.

Several different cell types have been transfected in vitro with the human *AAT* gene including canine hepatocytes,²² sheep endothelium,²³ and cotton rat airway epithelium.²⁴ In each case reasonable levels of transfection ranging from 25% to 90% of cells was achieved with production of human *AAT* mRNA and secretion of protein. In some of these studies protein function was shown as inactivation of neutrophil elastase. Hepatocytes transfected with retroviruses *ex vivo* have been retransplanted into dogs and human *AAT* mRNA detected for up to 47 days; low levels of protein production could be shown for 14 days.²² In sheep, segments of ligated vessels perfused with adenovirus *AAT* cDNA for 15 minutes produced protein within the endothelial cells detectable for up to 14 days, but no human *AAT* could be detected in the circulating blood.²³ In the cotton rat direct instillation of adenovirus *AAT* cDNA into the lungs was followed by detection of the human protein in airway lining fluid, but only at 2% of the required level for function.²⁴ Finally, rabbits receiving liposome-AAT complexes, either by intravenous injection or nebulisation, demonstrated human *AAT* mRNA and protein in both liver and lungs, again at low levels.²⁵

Based on these studies one human gene therapy protocol has been submitted to the regulatory authorities in the USA. This will first assess whether topical application of liposome-AAT complexes into the nasal epithelium of subjects with α_1 -antitrypsin deficiency results in detectable mRNA and protein, and whether any histological changes are seen as a result of gene transfer. Subsequently, the study aims to deliver the complex into the lower airways of subjects scheduled for elective pneumonectomy. Three days before surgery the complex will be instilled bronchoscopically and the site of exposure marked with a dye. At the time of surgery the transfected area will be lavaged for assessment of α_1 -antitrypsin levels and the specimen examined for mRNA and protein.

Gene therapy for lung cancer

As for other tumours, a number of gene therapy strategies are potentially applicable to lung cancer. Increasing tumour immunogenicity by local expression of, for example, cytokines may lead to increased killing via cytotoxic T lymphocytes. Furthermore, overexpression of HLA antigens such as B7 on the tumour cells may produce similar effects. Both of these strategies can be achieved by removal of tumour cells with *ex vivo* transfection with the appropriate gene and, following irradiation, reimplantation of these cells at the tumour site. Direct *in vivo* gene transfer into the tumour is an alternative means of gene delivery. The interleukin 4 gene has been successfully introduced into human non-small cell (NSCC) tumour cells in vitro using retroviruses.²⁶ Murine lung cancer cells transfected with the interferon- γ gene have been shown to reduce the number of established metastases in a mouse model.²⁷ A human trial of liposome-mediated interleukin 2 gene transfer for small cell lung cancer has been submitted to the regulatory authorities in the USA.

A second strategy is to upregulate the production of tumour suppressor genes. *p53* is a protein involved in DNA repair and synthesis, and mutations in this gene have been shown to be an independent poor prognostic factor in lung

cancer.²⁸ *p53* mutations are estimated to be present in approximately 50% of all types of lung tumours. Retroviral or adenoviral introduction of the *p53* gene into NSCC in vitro reduced growth rate by up to 80%, but only in cell lines with *p53* mutations.²⁹ Furthermore, in nude mice injected intratracheally with tumour cells, 75% developed tumours. However, in mice injected three days later with adenoviral *p53* constructs this progression only occurred in 25%. Recently a proposal for a human trial to express *p53* in NSCC has been given approval in the USA.

An opposite approach is the downregulation of oncogene expression. The *K-Ras* gene produces a protein p21 involved in cellular signal transduction. Approximately 30% of lung adenocarcinomas have mutations in the *K-Ras* gene, and again this is an independent poor prognostic factor in these cases.³⁰ Transfection of the *K-Ras* antisense gene into NSCC in vitro produces up to 10-fold growth inhibition, but only in *K-Ras* mutant cell lines. Again with intratracheal tumour cell injection into nude mice, *K-Ras* antisense reduced tumour formation from 90% to 13%.³¹ A proposal for introduction of *K-Ras* antisense into human NSCC has been submitted.

Finally, introduction of "suicide" genes has been suggested as treatment for many types of cancer. One example is the thymidine kinase (TK) enzyme, the viral but not the mammalian form of which metabolises ganciclovir to its monophosphate. The mammalian form metabolises the monophosphate to the triphosphate which competes with nucleotides for DNA replication leading to cell death. Thus, introduction of the viral form of the *TK* gene renders cells susceptible to ganciclovir. Furthermore, an important phenomenon known as the "bystander effect" means that not all tumour cells need to be transfected. As long as about 10% of cells within a tumour are transfected, the surrounding non-transfected cells are also killed by ganciclovir; the most likely explanation of this is cell to cell passage of the ganciclovir monophosphate. The ganciclovir concentration needed for cell killing is approximately that achieved in the treatment of cytomegalovirus infection with this agent. Adenovirus-mediated herpes simplex *TK* has been used to transfect human mesothelioma cells in vitro, and subsequent administration of ganciclovir produced virtually 100% cell death.³²

Clearly, one of the major challenges for gene therapy of lung cancer using any of these potential techniques is the ability to target metastatic diseases in addition to the initial tumour mass. Given the current difficulties with gene transfer into any cell type in vivo, such selective targeting suggests that gene therapy for lung cancer will be the most difficult goal of the three diseases outlined above.

Conclusions

At present the biggest hurdles for establishing gene therapy in clinical practice are efficient in vivo gene transfer and the molecular knowledge of which genes are most usefully targeted in a given acquired disease. Many laboratories are trying to improve the former, and rapid progress is being made in the development of new gene transfer agents. The aim for respiratory diseases is to produce efficient targeted transfer of the gene or antisense oligonucleotide of choice into either the pulmonary epithelium or endothelium. If this can be achieved gene therapy for both inherited and acquired lung diseases is likely to be a realistic therapeutic option in the future.

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