J. Pharm. Pharmacol. 1995, 47: 351–354
Received December 7, 1994
Accepted December 7, 1994

© 1995 J. Pharm. Pharmacol.

Towards Gene Therapy For Cystic Fibrosis*

E. W. F. W. ALTON

Ion Transport Unit, Department of Thoracic Medicine, Emmanuel Kaye Building, Manresa Road, London SW3 6LR, UK

Cystic fibrosis (CF) is the commonest lethal inherited disease in the Caucasian population. Approximately 1 in 20 people are heterozygous for the abnormal gene, and since it is inherited with autosomal recessive characteristics, approximately 1 in 2000 live births in the UK are children with CF. Some 50 years ago the mean life expectancy was just one year, but this has now risen in the UK to approximately 30 years. The principal pathology centres around the respiratory and intestinal tracts. In both cases these hollow epithelial lined organs become filled with thickened tenacious secretions. This is particularly important in the airways where bacterial colonization leads to recurrent chest infections and eventually respiratory failure. The intestinal symptoms are generally milder and of two types. First, those resulting from blockage in a manner analagous to that in the airways (meconium ileus). This occurs in approximately 10% of CF neonates and can generally be treated without the need for surgery. Secondly, malabsorption of gut contents resulting from blockage and atrophy of the pancreatic ducts and hence reduced secretion of the pancreatic enzymes needed for absorption. Again, this can be improved through the use of pancreatic supplements. Thus, the principal problem which needs to be addressed is the prevention of lung damage.

The CF gene is present on the long arm of chromosome 7 and was cloned in 1989 (Riordan et al 1989). The protein coded for, so called cystic fibrosis transmembrane conductance regulator (CFTR), functions as a chloride channel in the apical membrane of respiratory and intestinal epithelial cells. Elevation of the second messenger cAMP within the cell results in opening of the channel to allow chloride secretion from the cell on to the mucosal surface. Water will follow this chloride movement by osmosis, and this provides a means by which this surface can be hydrated. In turn this is likely to be important in the airways in the process of mucociliary clearance. Thus, inhaled particles and bacteria are removed from the airways by the synchronized beating of cilia on the mucosal surface of the epithelial cells. These cilia beat in a thin fluid layer which allows for maximal efficiency of this process. Chloride secretion is likely to provide an important contribution to this layer.

In patients with CF, mutations in the CFTR gene result in reduced or absent cAMP-mediated chloride secretion in all affected organs including the airways (Welsh 1990). This is because the abnormal protein is either mislocalized, never reaching the mucosal surface, or if it does reach its intended target functions with markedly reduced efficiency. The likely net result is that water movement is also reduced leading to a suboptimal periciliary layer in the airways. In turn this will result in impaired mucociliary clearance, reduced clearance of bacteria and the resulting repeated infections which predispose to lung damage. Sodium absorption from the airway surface is also abnormal in these patients, being increased 2–3-fold. The link between the former basic defect and the secondary sodium abnormality is presently unclear. However, since water will again follow the sodium movement, this second abnormality will also tend to dehydrate the airway surface liquid exacerbating the problem.

One potential therapeutic approach is to administer the sodium-channel blocker amiloride to the airways of these patients. Two studies have addressed this issue with conflicting results. In the first (Knowles et al 1990) all conventional treatment for CF respiratory symptoms was stopped and either amiloride or placebo administered in a double-blind placebo controlled study. In these adult patients with previously stable lung function, both groups showed a decline, with the amiloride-treated group showing a significantly smaller reduction in FEV_1. Our own 6-month double-blind placebo controlled study (Graham et al 1993) showed no additional benefit of amiloride over and above existing treatments. A large multi-centre trial of amiloride is underway in the USA and should help to determine the usefulness, if any, of this form of treatment for CF.

A second possible pharmacological approach is to try to bypass the defective CFTR chloride channel and up-regulate other types of chloride channel present in the apical membrane of airway epithelial cells. In particular, ATP or UTP have been suggested to be of potential interest (Knowles et al 1991). Both these agents increase intracellular levels of calcium and open a chloride channel distinct from CFTR in airway epithelium. They also have small effects on increasing mucociliary clearance and will shortly be assessed (in combination with amiloride) in a clinical trial.

However, the most obvious, and perhaps elegant, way of treating CF would be to introduce a new normal copy of the gene into the respiratory tract of these patients. Following the identification of the CF gene this has become a realistic possibility, and this was given further recent impetus with the production of several mouse models for CF (Dorin et al 1992; Snouwaert et al 1992; O'Neal et al 1993; Ratcliff et al 1993). We (Alton et al 1993), and others (Hyde et al 1993), have therefore assessed whether CFTR gene transfer into these CF mice could correct the ion transport abnormalities present. We devised a nebulizer system for delivering agents

*Presented at the British Pharmaceutical Conference in London, September 18, 1994.

to the respiratory tract of mice. This was validated using the dye methylene blue as well as the reporter gene β-galactosidase, demonstrating that up to approximately 40% of lower airway epithelial cells could be transfected using the cationic liposome DC-cholesterol : DOPE as the gene transfer agent. We also devised a technique for delivering DNA : liposome complexes to the large intestine of these animals. Again this was validated using methylene blue; given the high levels of endogenous β-galactosidase present in this organ, reporter gene validation was not undertaken.

Subsequently, *CFTR* cDNA-DC cholesterol : DOPE complexes were administered to exon 10 insertional CF mice and assays for mRNA and correction of the ion transport defects undertaken. With regard to the former, each of the eight treated animals showed evidence for expression of human specific *CFTR* mRNA; no expression was seen in the sham-treated or untreated animals. All assays were conducted in a blinded manner with respect to the treatment received. Importantly, however, there was marked variability, both within and between animals, with respect to *CFTR* mRNA expression. In-vivo analysis of the nasal ion transport properties of the treated animals showed that there was restoration of approximately 50% of the deficit between CF and wild-type animals in the chloride abnormality. Interestingly, no improvement was seen in the sodium defect. As for the mRNA studies, marked variability was seen, some animals showing complete correction of the chloride defect and others little or none. Very similar results were seen in the trachea when studied in Ussing chambers in-vitro. Again there was restoration of approximately 50% of the deficit in the chloride abnormality. The exon 10 insertional mouse model does not show the increased sodium transport seen in man in the lower airways; rather this model demonstrates reduced transport of this ion. This abnormality was also improved towards normal values following gene transfer. As in the upper airways marked variability was seen.

With respect to the intestinal tract, the jejunum serves as a useful control tissue since the DNA : liposome complex will not reach this region of the intestinal tract. As expected no changes in the abnormal ion transport present in this region in the CF mice were seen. In contrast there was a modest, approximately 20% increase in chloride secretion towards wild-type values in the distal ileum and large intestine. Importantly, no signs of toxicity related to administration either in the respiratory or intestinal tract were seen.

This study and a similar one from the Oxford/Cambridge group (Hyde et al 1993) suggested that liposome-mediated *CFTR* gene transfer may be a practical option for initial studies of gene transfer into CF patients. We, therefore, undertook a number of preparatory studies in man in-vivo to assess the feasibility of such a trial. We first assessed the safety aspects of nasal administration of DC-cholesterol : DOPE in six non-CF and three CF subjects (Middleton et al 1994a). Nasal potential difference provides a good index of tissue integrity and we, therefore, studied both sodium and chloride transport in these subjects before, and 1 and 5 days following, intranasal administration of the dose of liposome likely to be used in a study of gene transfer. No alterations were seen in any parameter in either group of subjects. Furthermore, no changes in spirometry resulted from this administration. Finally, we assessed whether the presence of liposomes would alter the growth or sensitivity of the resident bacteria present in the sputum of CF subjects. Again no significant effects were seen, suggesting that topical administration of DC-cholesterol may not produce deleterious effects in-vivo in man.

To assess the effects of *CFTR* gene transfer in man in-vivo it was important to have in place a technique for the measurement of both the sodium and chloride defects. We, therefore, next assessed the optimal protocol for such a study (Middleton et al 1994b). In six CF subjects we compared the signal for chloride secretion produced either by perfusion of the β-agonists isoprenaline or terbutaline, or by perfusion of a low chloride-containing solution. A combination of sequential perfusion with low chloride followed by isoprenaline provided the optimal discrimination between CF and non CF-subjects. With respect to sodium measurements, both the baseline PD as well as subsequent perfusion with amiloride provided a good measure of the absorption of this ion. This protocol is likely to be used in many of the forthcoming gene therapy trials for this disease.

With these studies in place, we began the series of steps necessary to gain ethical permission for a human trial of liposome-mediated gene transfer to the nasal epithelium of CF patients in 1992 (Caplen et al 1994). Essentially, four stages had to be gone through beginning with the national ethical watch-dog, the Clothier Committee (now chaired by Professor Dame June Lloyd). The safety aspects of the therapeutic product were assessed by the Medicines Control Agency, whilst the well-being of the investigators was supervised by the Advisory Committee on Gene Modification. Finally, the study was assessed by our local Ethical Committee. One of the important aspects of this process was that we were the first group in the UK to test these various hurdles. For this reason we often found ourselves in the position of suggesting suitable guidelines in addition to trying to meet the various committees' requirements.

We obtained permission for the trial in early 1993 and then tackled the next hurdle of producing sufficient quantities of both DNA and liposome. For the former we are indebted to the Human Genome Resource Centre at Northwick Park who rapidly and efficiently produced the required amounts of DNA. With respect to the latter we chose to use DC-cholesterol : DOPE for two reasons. Firstly, we had obtained experience using this liposome in the CF mouse study; secondly, the Federal Drug Administration as well as the Recombinant Advisory Committee in the USA has approved the use of this agent for a human melanoma gene therapy trial conducted by Dr Gary Nabel. Professor Leaf Huang at the University of Pittsburgh, USA, who first produced DC-cholesterol (Gao & Huang 1991), therefore agreed to produce the large quantities of liposome needed for the trial.

We began recruitment in July 1993, and studied 15 homozygous delta 508 CF subjects, all pancreatic insufficient in a double-blind placebo controlled trial (Caplen et al 1995). We used a CFTR cDNA driven by a SV40 promoter complexed with DC-cholesterol in a ratio of 1:5 as in our study in CF mice. For ethical reasons we chose severely affected individuals with an $FEV_1 < 40\%$ predicted.

Further, because of the theoretical risk of germ line transmission, and because CF males are infertile, we only studied male subjects in this trial. The study was conducted in two stages. Three subjects were randomized to low dose DNA (10 μg per nostril) and three to medium dose (100 μg per nostril). A further three subjects received only liposome equivalent to that received by the medium-dose group. These nine subjects were studied, the code broken and safety assessed. We then proceeded to the second stage in which three subjects were randomized to high dose DNA (300 μg per nostril) and a further three who received the increased dose of liposome alone. Application to both nostrils was through a commercially available pump spray which produced an aerosol of mass median diameter 60 μm. Because of concerns that contact time with the nasal epithelium was an important factor in the efficiency of gene transfer, and because nasal mucociliary clearance is of the order of 10–15 min, we chose to administer the DNA-liposome complex in divided doses. For stage 1 this was every 15 min over 2·5 h and for stage 2, because of the much larger volumes, every 10 min over approximately 7 h. No side-effects related to the administration procedure were noted.

To study safety we assessed the patients clinically, asked them to keep a diary card and measured spirometry on each of the days we saw the subjects. These were 14 and 7 days before, and on the day of gene transfer. Subsequently, we studied them at days 1, 3, 4, 5, 7, 11, 18 and 28 following administration. In addition we took chest radiographs and a large series of blood tests at the beginning and end of the study period. Blood samples were also assessed on days 1 and 4 following gene transfer, looking respectively for acute toxicity and changes following our prediction for maximal gene expression. We also took a nasal biopsy from one nostril on day 4 for histological assessment. With regard to efficacy, half of this biopsy as well as nasal brushings taken on the same occasion were assessed for vector-specific mRNA using conventional RT-PCR techniques. Further, we measured nasal PD using the protocol outlined above on each of the days except day 4, when the nasal biopsy was taken.

There were no clinical abnormalities seen, nor changes on the radiographs or in the blood tests. The histological assessment was performed in a blinded manner and no differences were seen in the DNA or liposome groups with respect to conventional histology, neutrophil elastase, macrophage number or CD45+ T-lymphocytes. One subject, who received low dose DNA, showed a chronic subepithelial lymphocytic infiltrate. It is likely that if sufficient biopsies are taken from these patients with chronic nasal infection, such changes will be revealed; however, we cannot disprove that this related to the treatment received.

Of the nasal brushings only one was positive for plasmid DNA (not mRNA); this was from a high dose subject. Interestingly, however, five of the eight available biopsies from the DNA-treated subjects were positive both for DNA and mRNA. To summarize the measurements of nasal PD, the response to the low chloride perfusion showed a significant increase of approximately 20% from CF towards non-CF values. In some cases these reached normal values, but overall there was marked variability. The response to isoprenaline was virtually unchanged following gene transfer. Responses related to sodium transport were less marked than for chloride, although just detectable. The changes in the ion-transport parameters were maximal around day 3 and had returned to pretreatment values by day 7 following gene transfer.

It is perhaps worth considering these data in the context of the other CF gene therapy trials being undertaken. No other liposome-based study has yet started, although three others have received permission to proceed (Alabama, USA, Edinburgh and Oxford/Cambridge, UK). Six trials of adenoviral-mediated CFTR gene transfer are also underway at the National Institute of Health (NIH) Cornell, Iowa, Pennsylvania, North Carolina and Cincinatti in the USA and at Transgene in France. Two of these have reported preliminary findings. The first of these was the Iowa group (Zabner et al 1993) who looked at three subjects in a non-blinded uncontrolled study. They showed correction of both the sodium and the chloride abnormalities in the nasal epithelium for a period of approximately 2–3 weeks following single application. Conventional RT-PCR failed to show the presence of mRNA, but a modification of this process could demonstrate the appropriate message in two of the three subjects. The study by Crystal et al (1994) at the NIH assessed both nasal and lower airway application in four subjects. No measures of CFTR function were included, but mRNA was present in one of the four nasal specimens and CFTR protein in one of the nasal and one of the bronchial specimens. However, one of the patients (who received the highest dose) developed hypotension, fever and respiratory symptoms suggestive of an inflammatory reaction within the lungs. These changes were suggested to relate to an increase in interleukin-6, and resolved completely over a period of one month; thus, these adenoviral studies are beginning to define a therapeutic window with respect to dose-efficacy-safety considerations. It is likely that many of the problems encountered in these studies relate to immunological reactions focused on the viruses' coat proteins, and strenuous efforts are being made to prevent the virus from producing these proteins. It is likely, therefore, that safer adenoviral-mediated gene transfer will be possible with these second generation viruses. A further potential problem with their use is the effect of neutralizing antibodies in limiting the efficacy of repeated administration. Whilst this has yet to be studied in human trials, it has clearly been demonstrated in some, if not all, animal studies. This is of relevance since neither liposome- nor adenovirus-mediated gene transfer will produce stable integration of the gene into the genome and repeated administration will be needed.

With regard to efficacy most groups agree that there are similar problems to those seen in our CF mouse study. Thus, gene transfer is very variable and, despite relatively large quantities of DNA, is fairly inefficient. Additionally, chloride-related abnormalities appear easier to correct than the sodium defect. The clinical relevance of these findings is presently difficult to judge. Whether the chloride or sodium defect is the more important to correct is unknown. Further, the degree of correction needed for clinical benefit is also uncertain. However, recent studies suggest that approximately 5% of cells corrected may be

sufficient to produce normal ion transport properties within an epithelial sheet. Whilst clearly transfection efficiency needs to be improved, we may not be very far from the necessary in-vivo requirement.

With regard to future studies we have submitted an application to proceed with a lung trial on a similar basis to the nasal studies described above. This is principally to assess safety and also to learn how to measure gene transfer efficacy in this more difficult part of the airways. In parallel with this we are undertaking in-vitro laboratory studies to try to improve transfection efficiency using liposomes. Promising ideas will then be taken through a further nose trial before use in the lungs of CF patients. During this time further data will become available on the use of adenovirus for gene transfer. It is likely that neither of the present systems will be ideal for large phase III studies in man, and a combination of the two may eventually provide a better gene transfer agent.

References

Alton, E. W. F. W., Middleton, P. G., Caplen, N. J., Smith, S. N., Steel, D. M., Munkonge, F. M., Jeffery, P. K., Geddes, D. M., Hart, S. L., Williamson, R., Fasold, K. I., Miller, A. D., Dickinson, P., Stevenson, B. J., McLachlan, G., Dorin, J. R., Porteous, D. J. (1993) Non-invasive liposome-mediated gene delivery can correct the ion transport defect in cystic fibrosis mutant mice. Nature Genetics 5: 135–142

Caplen, N. J., Kinrade, E., Chakera, A., Coutelle, C., Williamson, R., Gao, X., Huang, L., Schorr, J., Porteous, D. J., Alton, E. W. F. W., Geddes, D. M., Hayes, P., Eleswarapu, R., Fisher, G., Gibson, K., Gilchrist, C. (1994) Gene therapy for cystic fibrosis in humans by liposome mediated DNA transfer: the production of resources and the regulatory process. Gene Therapy 2: 1–9

Caplen, N. J., Alton, E. W. F. W., Middleton, P. G., Dorin, J. R., Stevenson, B. J., Gao, X., Durham, S. R., Jeffery, P. K., Hodson, M. E., Coutelle, C., Huang, L., Porteous, D. J., Williamson, R., Geddes, D. M. (1995) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Nature Medicine 1: 39–46

Crystal, R. G., McElvany, N. G., Rosenfeld, M., Chu, C.-S., Mastrangeli, A., Hay, J. G., Brody, S. L., Jaffe, H. A., Eissa, N. T., Danel, C. (1994) Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nature Genetics 8: 42–51

Dorin, J. R., Dickinson, P., Alton, E. W. F. W., Smith, S. N., Geddes, D. M., Stevenson, B. J., Kimber, W. L., Fleming, S., Clarke, A. R., Hooper, M. L., Anderson, L., Beddington, R. S. P., Porteous, D. J. (1992) Cystic fibrosis in the mouse by targeted insertional mutagenesis. Nature 359: 211–215

Gao, X. A., Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commun. 179: 280–285

Graham, A., Hasani, A., Alton, E. W. F. W., Martin, G. P., Marriott, C., Hodson, M. E., Clarke, S. W., Geddes, D. M. (1993) No added benefit from nebulised amiloride in patients with cystic fibrosis. Eur. Resp. J. 6: 1243

Hyde, S. C., Gill, D. R., Higgins, C. F., Tresize, A. E., MacVinish, L. J., Cuthbert, A. W., Ratcliff, R., Evans, M. J., Colledge, W. H. (1993) Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. Nature 362: 250–255

Knowles, M. R., Church, N. L., Waltner, W. E., Yaankaskas, J. R., Gilligan, P., King, M., Edwards, L. J., Helms, R. W. B., Boucher, R. C. (1990) A pilot study of aerosolised amiloride for the treatment of lung disease in cystic fibrosis. N. Engl. J. Med. 322: 1189–1194

Knowles, M. R., Clarke, L. L., Boucher, R. C. (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. N. Engl. J. Med. 325: 533–538

Middleton, P. G., Caplen, N. J., Gao, X., Huang, L., Gaya, H., Geddes, D. M., Alton, E. W. F. W. (1994a) Nasal application of the cationic liposome DC-Chol: DOPE does not alter ion transport, lung function or bacterial growth. Eur. Resp. J. 7: 442–445

Middleton, P. G., Geddes, D. M., Alton, E. W. F. W. (1994b) Protocols for in vivo measurement of the ion transport defects in cystic fibrosis nasal epithelium. Eur. Resp. J. 7: 2050–2056

O'Neal, W. K., Hasty, P., McCray, P. B., Casey, B., Rivera-Pereez, J., Welsh, M. J., Beaudet, A. L., Bradley, A. (1993) A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. Hum. Molec. Genet. 2: 1561–1569

Ratcliff, R., Evans, M. J., Cuthbert, A. W., MacVinish, L. J., Foster, D., Anderson, J. R., Colledge, W. H. (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. Nature Genetics 4: 35–41

Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Ianuzzi, M. C., Collins, F. S., Tsui, L.-C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245: 1066–1073

Snouwaert, J. N., Brigman, K. K., Latour, A. M., Malouf, N. N., Boucher, R. C., Smithies, O., Koller, B. H. (1992) An animal model for cystic fibrosis made by gene targeting. Science 257: 1083–1088

Welsh, M. J. (1990) Abnormal regulation of ion channels in cystic fibrosis epithelia. FASEB J. 4: 2718–2725

Zabner, J., Couture, L. A., Gregory, R. J., Graham, S. M., Smith, A. E., Welsh, M. J. (1993) Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with CF. Cell 75: 207–216