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The effect of mucolytic agents on gene transfer across a CF sputum barrier in vitro

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Trials of gene transfer for cystic fibrosis (CF) are currently underway. However, direct application to the airways may be impeded by the presence of airway secretions. We have therefore assessed the effect of CF sputum on the expression of the reporter gene β-galactosidase complexed with the cationic liposome DC-Chol/DOPE in a number of cell lines in vitro. Transfection was markedly inhibited in the presence of sputum; the effect was concentration dependent and was only partially ameliorated by removal of sputum with phosphate-buffered saline (PBS) washing before gene transfer. However, treatment of the sputum-covered cells with recombinant human DNase (rhDNase, 50 μg/ml) but not with N-acetylcysteine, Nacystelyn, lysine (all 20 mM) or recombinant alginase (0.5 U/ml) significantly ($P < 0.005$) improved gene transfer. Adenovirus-mediated gene transfer efficiency in the presence of sputum was similarly inhibited, and again, treatment with rhDNase before transfection significantly improved gene transfer ($P < 0.005$). Transfection of Cos 7 cells in the presence of exogenous genomic DNA alone demonstrated similar inhibition to that observed with sputum and was also ameliorated by pre-treatment of DNA-covered cells with rhDNase. In a separate series of experiments performed in the absence of added sputum or genomic DNA, increasing concentrations of rhDNase resulted in a concentration-related decline in transfection efficiency. However, even at the highest concentration (500 μg/ml of rhDNase), transfection efficiency remained more than 50% of control. Thus, pre-treatment of CF airways with rhDNase may be appropriate before liposome or adenovirus-mediated gene therapy.

Keywords: *cystic fibrosis; gene therapy; sputum; rhDNase*

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease associated with premature death usually caused by persistent pulmonary infection, bronchiectasis and respiratory failure. The identification of the gene mutated in CF and the characterisation of the protein for which it codes – the cystic fibrosis transmembrane conductance regulator (CFTR)[1–3] – have been swiftly followed by the development of strategies for treating the disease with gene therapy. A number of phase I clinical trials are already reported[4–7] or underway, and all studies have focused on the respiratory tract as the target organ. While delivery of gene therapy topically to the airways of CF patients is likely to be the most efficient route, it is also likely that in the presence of established lung disease, gene transfer to the respiratory epithelium will be impeded by the sputum lining CF airways. The sputum may not only present an impenetrable barrier to the gene delivery system, limiting access to the underlying respiratory epithelial cells, but may also affect its integrity, either binding to complexes or inactivating them functionally. Whether sputum can effectively be removed before gene transfer remains unclear.

Other factors may also prejudice efficient gene transfer under these circumstances. Recombinant human deoxyribonuclease (rhDNase), known to reduce sputum viscoelasticity[8] and to improve lung function in both moderately and severely affected patients[9] is now widely prescribed for CF. rhDNase acts by cleaving neutrophil-derived DNA present in infected sputum. Its effect on exogenous DNA has not been studied but its presence in the airways represents a potentially adverse factor for efficient gene transfer. This raises the question of whether patients receiving rhDNase would be required to stop this treatment while receiving gene therapy or whether its advantageous mucolytic effects would weigh in favour of rhDNase being a useful adjunct for more efficient gene transfer.

In this study we have investigated the effect of freshly obtained CF sputum on the efficiency of gene transfer *in vitro* by transferring the reporter gene β-galactosidase (pCMVβ)[10] complexed with either the cationic liposome DC-Chol/DOPE[11] or incorporated in an adenoviral vector (AdCMVNLSβgal),[12] into epithelial cell lines maintained in culture in the presence or absence of sputum. We have studied whether *in vitro* gene transfer can be

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improved following removal of the sputum, first by simply washing with phosphate-buffered saline (PBS) and then by incubation with various mucolytic agents, comparing the effects of rhDNase with other mucolytics. Finally, we have studied gene transfer in the presence of exogenous genomic DNA to determine whether the inhibitory effect of CF sputum on gene transfer efficiency might be attributable to its high DNA content.

Results

The presence of sputum limits liposome-mediated gene transfer efficiency in vitro and this effect is concentration dependent

In the presence of fresh sputum derived from CF patients and diluted 1:1 in OptiMEM, transfer of β-galactosidase complexed with the cationic liposome DC-Chol/DOPE ($n = 6$) to Cos 7 cells was markedly reduced compared with control cells transfected in the presence of OptiMEM alone (Figure 1, solid bars). This effect was concentration dependent. In the presence of sputum diluted 1:10, β-galactosidase transfer remained at a similar level to control but improved significantly ($P < 0.005$) by the time the sputum was diluted to 1:10 000. Even at this dilution, however, transfection efficiency did not reach control levels. Removal of the sputum from the cells by washing with PBS before gene transfer did not result in significant amelioration of this effect (Figure 1, open bars).

Removal of sputum with rhDNase but not with other mucolytic agents significantly improves gene transfer efficiency

Compared with simple PBS washing of cells covered with sputum (diluted 1:10) before transfection, efficiency of gene transfer to Cos 7 cells was significantly ($P < 0.005$) improved when the sputum-covered cells were pre-incubated with rhDNase (50 μg/ml) (Figure 2a). Compared with PBS, however, there was no significant improvement following pre-incubation with *N*-acetylcysteine, Nacystelyn, lysine (each 20 mm) or with recombinant alginate lyase (0.5 U/ml). Similarly, transfer of β-galactosidase to the human bronchial epithelial cell line 16HBE14o–, (Figure 2b) and to the CF submucosal epithelial cell line 2CFSMEo– (Figure 2c) was markedly inhibited in the presence of sputum and not significantly improved following washing of the cells with PBS before gene transfer. Transfection efficiency was again significantly ($P < 0.005$) improved following washing of the cells with rhDNase but not with other mucolytic agents before transfection.

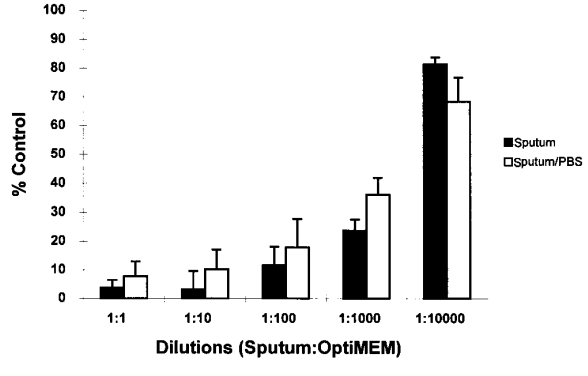

Figure 1 *The effect of sputum on liposome-mediated gene transfer efficiency. The reporter gene β-galactosidase complexed with the cationic liposome DC-Chol/DOPE was transferred to Cos 7 cells either in the absence (control) or presence of increasing dilutions of freshly obtained CF sputum (solid bars). Data are presented as % control (mean ± s.e.m., n = 6 patients' sputum samples) representing cells transfected in the presence of medium alone and which expressed 326.3 ± 25.2 pg β-galactosidase/μg of protein following gene transfer. In separate studies the cells were incubated for 60 min with the same serial dilutions of sputum which were removed by washing with PBS and replaced with OptiMEM before gene transfer (open bars). Data are again expressed as % control (mean ± s.e.m., n = 6) where control cells expressed 411.5 ± 76.0 pg β-galactosidase/μg of protein.*

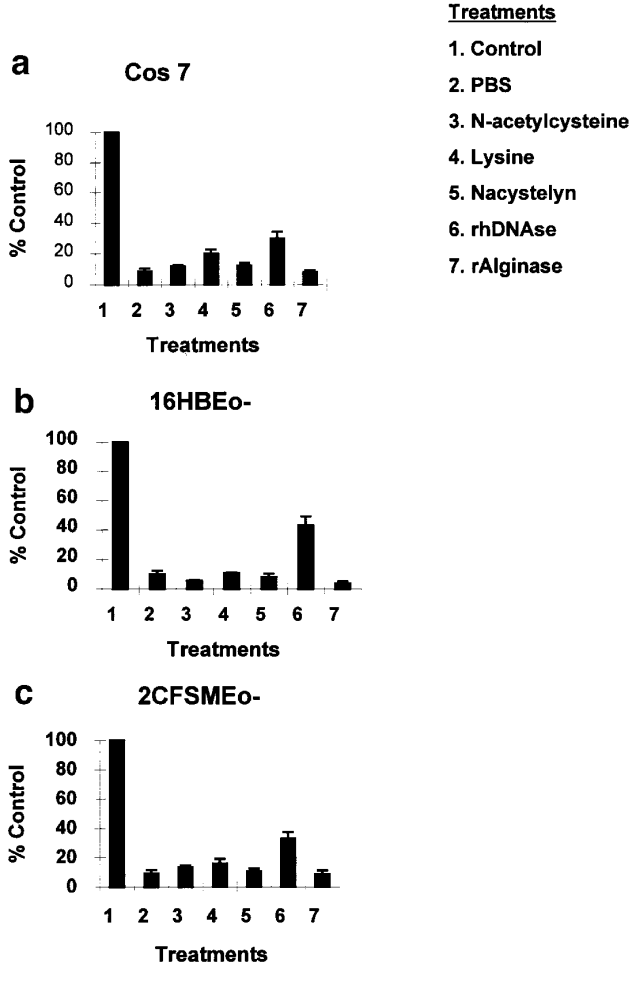

Figure 2 *The effect of mucolytic agents on liposome-mediated gene transfer efficiency in cells pretreated with CF sputum. Cos 7 cells (a), 16HBE14o– cells (b) and 2CFSMEo– cells (c) were incubated with sputum (1:10 dilution in OptiMEM), washed three times with PBS and incubated with one of the mucolytic agents before gene transfer. Compared with PBS washing alone, efficiency of gene transfer into all three cell lines was significantly ($P < 0.005$) improved following incubation with rhDNase (50 μg/ml). Compared with PBS, however, there was no significant improvement following removal of sputum with N-acetylcysteine, Nacystelyn, lysine (each 20 mm) or recombinant alginate lyase (0.5 U/ml). Data are expressed as % control (mean ± s.e.m., n = 6) where control was 697.8 ± 206.6, 223.6 ± 11.3 and 385.2 ± 26.2 pg β-galactosidase/μg protein for Cos 7 cells, 16HBE14o– cells and 2CFSMEo– respectively.*

The reporter gene β-galactosidase complexed with the cationic liposome DC-Chol/DOPE remains functional in the presence of rhDNase

Transfection of Cos 7 cells in the presence of increasing concentrations of rhDNase resulted in a concentration-related decline in transfection efficiency, although even at the highest concentration used, transfection efficiency remained more than 50% of control (Figure 3).

The presence of sputum also limits the efficiency of adenovirus-mediated gene transfer in vitro and this effect is similarly abrogated by washing of the sputum with rhDNase

Transfer of β-galactosidase complexed with the adenoviral vector AdCMVNLS to the three cell lines, Cos 7, 16HBEo– and 2CFSMEo–, was limited in the presence of fresh CF sputum, and again this effect was concentration dependent (Figure 4). In all three cell lines, incubation of the sputum-covered cells with rhDNase (50 μg/ml) before transfection significantly ($P > 0.05$) improved transfection efficiency compared with control cells transfected in the presence of sputum without rhDNase. In the case of the 16HBEo– cells, rhDNase treatment resulted in transfection efficiency that was significantly ($P > 0.05$) higher than that seen in control cells transfected in the absence of sputum.

Gene transfer efficiency in vitro is inhibited in the presence of exogenous genomic DNA. This effect is concentration dependent and ameliorated by incubation of the DNA-covered cells with rhDNase before transfection

Transfection of Cos 7 cells in the presence of increasing concentrations of exogenous genomic DNA resulted in a concentration-dependent decline in transfection efficiency (Figure 5). At the highest concentration used (500 μg/ml), transfection efficiency was inhibited to less than 10% of control cells transfected in the absence of exogenous DNA. At a concentration of 1 μg/ml, however, there was no significant difference between β-galactosidase expression in cells transfected in the presence or absence of exogenous DNA. Preincubation of DNA-covered cells with a single dose of hrDNase (50 μg/ml) resulted in significant retrieval of transfection efficiency at all DNA concentrations greater than 1 μg/ml.

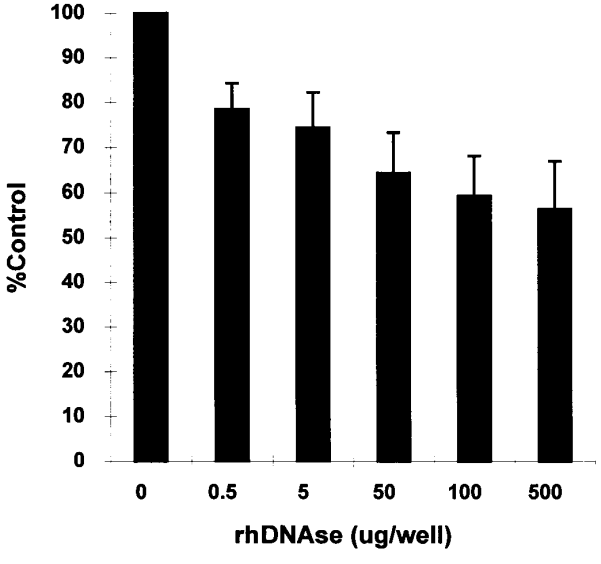

Figure 3 *The effect of rhDNase on the functional integrity of the gene–liposome complex. Transfection of Cos 7 cells in the presence of increasing concentrations of rhDNase (n = 6) resulted in a concentration-related decline in transfection efficiency. At the highest concentration of rhDNase transfection efficiency was significantly lower ($P < 0.005$) but nevertheless remained greater than 50% of control. Data are expressed as % control (mean ± s.e.m., n = 6) where control was 365.9 ± 18.8 pg β-galactosidase/μg protein.*

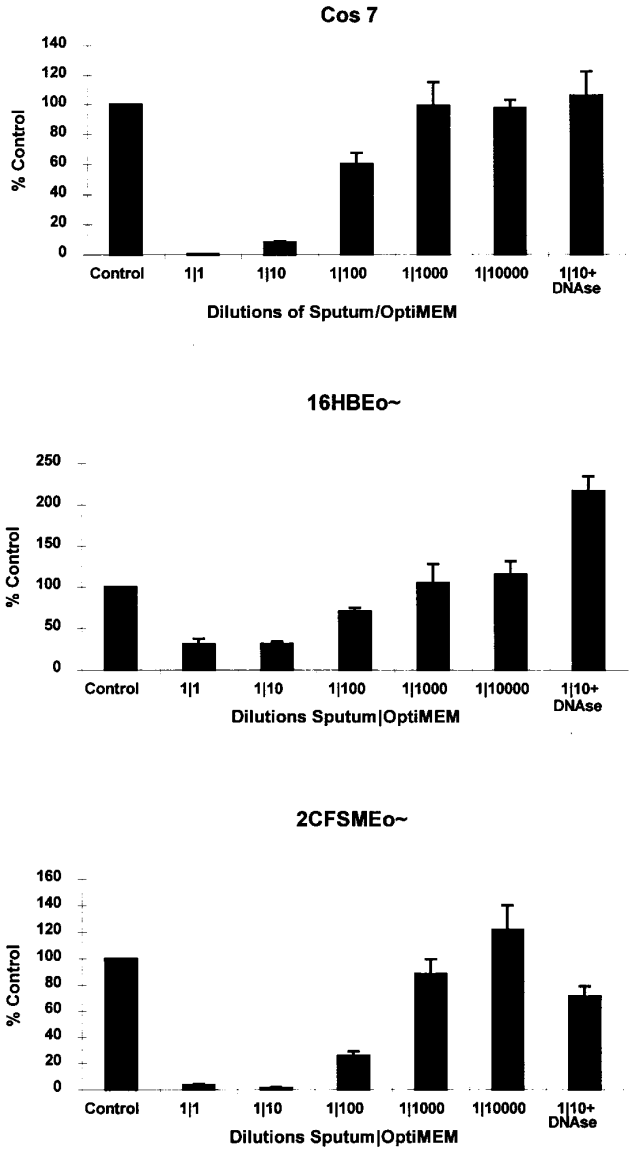

Figure 4 *The effect of sputum on adenovirus-mediated gene transfer efficiency with and without pretreatment of the cells with rhDNase. Transfer of the reporter gene β-galactosidase complexed with the adenoviral vector AdCMVNLS was assessed in the three cell lines Cos 7, 16HBEo– and 2CFSMEo–, respectively in the absence (control) or presence of increasing dilutions of freshly obtained CF sputum. Cells covered in sputum diluted 1:10 in OptiMem were also transfected following incubation of the sputum-covered cells with rhDNase for 60 min and demonstrate reversal of the inhibition seen when transfection took place at the same dilution of sputum but in the absence of rhDNase. Data are expressed as % control (mean ± s.e.m.) where control cells expressed 328.7 ± 36.8 (Cos 7), 329.1 ± 32.1 (16HBEo–) and 41.5 ± 4.81 (2CFSMEo–) pg β-galactosidase/mg protein, respectively.*

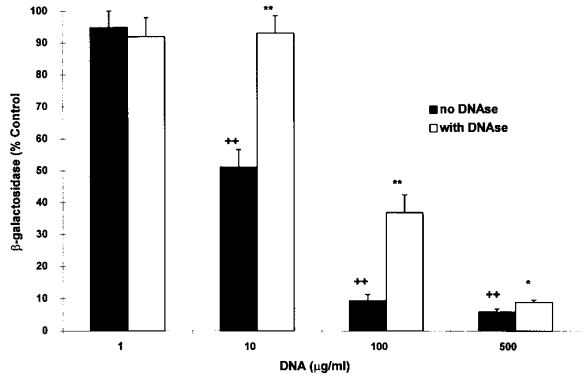

Figure 5 *The effect of genomic DNA on liposome-mediated gene transfer efficiency with and without pretreatment of the cells with rhDNase. The reporter gene β-galactosidase complexed with the cationic liposome DC-Chol/DOPE was transferred to Cos 7 cells either in the absence (control) or presence of increasing dilutions of genomic DNA (solid bars). At concentrations of 10, 100 and 500 μg/ml, the presence of exogenous DNA significantly ($P < 0.01^{++}$) inhibited transfection efficiency. Data are presented as % control (mean ± s.e.m., n = 6) representing cells transfected in the presence of medium alone and which expressed 895.3 ± 71.5 pg β-galactosidase/μg of protein following gene transfer. In a parallel study, incubation of the cells with the same dilutions of DNA was followed by incubation for 60 min with rhDNase (50 μg/ml) (open bars) before transfection. As depicted, reversal of inhibition of transfection efficiency was significant ($P < 0.01^{**}$) where 10 and 100 μg/ml of DNA had been present and also significant ($P < 0.05^{*}$) where 500 μg/ml of DNA had been present. Data are again expressed as % control (mean ± s.e.m., n = 6).*

Discussion

In this study we have shown that the efficiency of both cationic liposome-mediated and adenovirus-mediated gene transfer to epithelial cells *in vitro* is markedly reduced in the presence of CF sputum and that this effect is concentration related. Removal of the sputum from the cell monolayers by washing with PBS does not ameliorate the effect, suggesting that sputum not only presents a physical barrier but may bind to cell surface structures thereby limiting gene transfer. Removal of sputum with the mucolytic agent rhDNase, however, significantly improves transfection efficiency suggesting that the high DNA content in CF sputum is likely to play an important role in the inhibition of gene transfer efficiency. This possibility is strengthened by the demonstration that in the presence of genomic DNA alone, transfection is inhibited in a concentration-related manner which can be reversed by pre-incubation of the DNA-covered cells with rhDNase. Finally, despite the potential for rhDNase-induced degradation of the transgene, plasmid DNA when complexed with either a cationic liposome or an adenoviral vector, retains functional integrity in the presence of rhDNase.

Gene therapy for CF is likely to be most effective if undertaken before the onset of lung damage and the chronic production of sputum. Nevertheless, in practice, the last few decades have witnessed a dramatic increase in the survival of patients with CF, attributable in large part to early diagnosis and intervention as well as advances in antibiotic therapy and nutritional support. Thus, the median survival has increased to approximately 30 years and currently more than a third of CF patients are over the age of 16. These are the patients, many of whom are colonised with *Pseudomonas aeruginosa* and produce large amounts of sputum, who are most likely to be the focus of clinical trials. Furthermore, they present the challenge of developing gene therapy not only to prevent lung disease but also to halt progression of established disease. It is therefore important to study the effects of sputum on gene transfer and to find ways of ameliorating these effects.

Mucus is known to act as a physical barrier by preventing passage of molecules >17 000 mol. wt. from reaching the epithelium.[13] The size of a DNA–liposome complex, even in its theoretically simplest conformation, is log orders greater than this (DNA approximately 650 mol. wt. per base pair (bp) and pCMVβ approximately 8600 bp; DC-Chol approximately 500 mol. wt., DOPE approximately 750 mol. wt. with DC-CHOL:DOPE complexed at a ratio approximately 1500:1400; thus the simplest complex would be approximately 7 350 000 mol. wt.). It is likely therefore that exogenous DNA would be excluded by a contiguous sheet of mucus unless the nature of the delivery system itself confers properties of altered permeability across the mucus. The macromolecular properties and architecture of CF mucus glycoproteins are essentially similar to mucins from normal respiratory secretions.[14] However, the additional load of bacteria and bacterial products such as pseudomonas-derived alginates, necrotic neutrophils and neutrophil-derived DNA are likely to contribute even further to the physical barrier presented by mucus. In the light of these considerations, the effect of UV sterilization of the sputum before the gene transfer experiments should be noted. It was not possible to perform these experiments using fresh, infected CF sputum without prior sterilization because early contamination of the cell cultures prevented further assessment of gene transfer. It is possible that exposure to UV light may destroy those components of sputum most likely to have adverse effects on gene transfer, for example, the high molecular weight components of sputum or the alginates produced by colonizing mucoid *Pseudomonas aeruginosa*.[15,16] Nevertheless, the fact that the sterilized sputum used in these experiments limited gene transfer efficiency makes it likely that untreated sputum may present an even greater barrier to gene transfer. Thus, this model may simulate a more optimum set of conditions than would be met *in vivo*.

Mucus glycoproteins are high molecular weight biopolymers where glycopeptides are linked together by disulfide bonds. The macromolecule contains 80% sugar residues and this allows the potential for hydrogen bonding between adjacent chains leading to the formation of a complex, three-dimensional viscoelastic gel. Agents such as *N*-acetylcysteine have the capacity to break disulfide bonds, whereas lysine will disrupt hydrogen (secondary) bonding. Both types of agents would be predicted to exert mucolytic activity and their combination as Nacystelyn has been shown to be synergistic *in vitro*.[17] Since neither agent alone nor the combination (ie Nacystelyn) increased gene transfer in these experiments, one conclusion is that the mucoglycoproteins in CF sputum, do not by themselves represent a significant barrier to cell access by the gene and its delivery vehicle.

Mucoid *Pseudomonas aeruginosa*, a common opportunistic pathogen in CF, produces large quantities of the exopolysaccharide alginate. Alginate is thought to preserve the bacterial eco-environment by protecting the organism from dehydration. It is also thought to play a

role in bacterial virulence through its antiphagocytic and immunosuppressive properties,[18–20] and has been implicated as a pseudomonal adhesin, facilitating increased binding of the organism to respiratory epithelium.[21–23] Thus, the alginase component of the sputum may bind to the epithelial cell surface and may be resistant to PBS washing before gene transfer. If this were the case, bacterial recombinant alginate lyase, shown *in vitro* to degrade alginate[24] and to reduce the adherence capacity of mucoid *Pseudomonas aeruginosa*,[25] may have been useful in disrupting this adherent layer. The negative results demonstrated above suggest that alginate is not primarily responsible for inhibition of gene transfer. However, it is possible that alginates may have been degraded during the UV sterilization of the sputum, masking their effects. Alternatively, it has previously been shown that the action of alginate lyase on the viscoelastic properties of CF sputum is limited [26] and this limitation has been attributed to inhibition of the enzyme by high levels of Zn^{2+} and/or Ca^{2+} present in CF sputum.

Extracellular DNA released by disintegrating inflammatory cells, particularly neutrophils, is present in infected sputum in high concentrations.[27–29] The cloning of the gene for human DNase I has been followed by the development of the recombinant human protein (dornase alpha) and has been shown *in vitro* to reduce the viscoelasticity and adhesiveness of CF sputum.[8] rhDNase is administered by aerosol inhalation and has been evaluated extensively for safety and efficacy in patients with CF $\geqslant$5 years with a baseline forced vital capacity $\geqslant$40% of predicted value.[9] Phase I, II and III clinical trials of nebulised rhDNase have demonstrated improved lung function and a reduction in the incidence of respiratory tract infectious exacerbations requiring parenteral antibiotics.[9] Consequently, it is now widely prescribed for CF, involving >50% of adult patients and approximately 15% of children with CF at the Royal Brompton Hospital.

Reversal of the inhibitory effect of CF sputum on gene transfer by rhDNase suggested that the high DNA content of the sputum was likely to play an important role in inhibition. To confirm this hypothesis, transfection was carrried out in the presence of increasing concentrations of exogenous DNA. In order optimally to simulate the components of CF sputum, genomic rather than plasmid DNA was used. The concentration range chosen was based on approximate measurements previously documented for CF sputum *in vivo* (approximately 0.6 mg/ml),[29] and took into account the 1:10 dilution of sputum used in the experiments described above. The inhibition of transfection demonstrable in the presence of genomic DNA proved similar to that seen when transfection was carried out in the presence of CF sputum and suggests that DNA plays an important role as a barrier to gene transfer *in vivo*.

By the nature of its action, we expected that concomitant use of rhDNase with gene transfer agents would be contraindicated, raising the possibility that rhDNase would have to be withdrawn from CF patients enrolled for clinical trials of gene transfer. This study, however, demonstrates a beneficial effect of rhDNase upon gene transfer efficiency across a sputum barrier, albeit only with partial restoration. The observations suggest, furthermore, that neutrophil-derived DNA may bind to epithelial cell surfaces in a manner that is not reversible by PBS washing and that it is this sputum component which consequently limits gene transfer – regardless of the gene delivery system.

Electrophoresis and Southern blotting have previously demonstrated that naked plasmid DNA is degraded in the presence of rhDNase, but when complexed with cationic liposomes, the DNA remained at least partially protected from this degradation (data not shown). This would explain how, in the presence of rhDNase, successful transfer of the reporter gene complexed with liposome remains possible. These observations are also congruent with documentation of the structures formed during the interaction of DC-Chol/DOPE and plasmid DNA.[30] These structures, studied by freeze–fracture electron microscopy, resemble 'meatball-spaghetti' assemblies including liposome complexes ('meatballs') and bilayer lipid-covered DNA ('spaghetti') connected to liposome complexes as well as occurring free in suspension. With respect to transfection, there is some evidence to suggest that the lipid-coated spaghetti-like structures may be the active DNA–liposome complex. The DNA encapsulation by the bilayer coating may well provide some protection from degradation by rhDNase. Whether transfection efficiency in the presence of rhDNase could be further improved to control levels by increasing the amount of DNA–liposome complex delivered remains to be elucidated.

In the case of adenovirus-mediated gene transfer, pretreatment of sputum-covered cells with rhDNase resulted in recovery of gene transfer efficiency to within the control range. These data suggest that here, the limitation of gene transfer efficiency was essentially due to the sputum barrier and that the transgene within its viral carrier remains fully protected from potential adverse effects of rhDNase.

In conclusion, we present an *in vitro* model for gene transfer across a sputum barrier in the lower airways of CF patients. Although this model cannot be directly comparable with *in vivo* gene transfer across a sputum barrier, the study demonstrates that purulent CF sputum has an adverse affect on gene transfer and that removal of the sputum may be a necessary prerequisite for gene therapy to the lower airways in patients with excess mucus production. Finally, the study suggests that mucolytic treatment with rhDNase may be appropriate before gene transfer into the airways, providing a useful adjunct to gene transfer efficiency regardless of whether cationic liposomes or an adenoviral vector is used for gene delivery.

Materials and methods

Cells

All media and supplements were obtained from Gibco BRL, Paisley, UK. The SV-transformed renal epithelial cell line Cos 7 (derived from African Green Monkey)[31] was maintained at 37°C/5%CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine and 1% penicillin/streptomycin. The human bronchial epithelial cell line (16HBE14o–)[32] and the CF submucosal epithelial cell line (2CFSMEo–)[33] were maintained in minimum essential medium (MEM) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% l-glutamine and non-essential amino acids.

Sputum

Fresh sputa were obtained from CF patients (n = 6 for each set of experiments) admitted to the Royal Brompton Hospital with an infective pulmonary exacerbation and who were known to be colonized with mucoid strains of *Pseudomonas aeruginosa*. The sputum was transferred to plastic Petri dishes (Greiner Labortechnik, Dursley, UK), sterilized by exposure to a short-wave UV light source for 30 min and then mixed with OptiMEM at ratios of 1:1, 1:10, 1:100, 1:1000, 1:10000 and 1:100000 (v:v).

Plasmid DNA

The eukaryotic expression plasmid pCMVβ (Clontech Laboratories, Palo Alto, CA, USA) containing the *Escherichia coli* β-galactosidase gene under the control of the cytomegalovirus immediate–early promoter and enhancer was used to assess transgene expression after cell transfection. Plasmid DNA was prepared as described previously.[34]

Cationic liposomes

DC-Chol was prepared using the method of Gao and Huang[11] and the cationic liposome DC-Chol/DOPE was prepared by microfluidisation and diluted to a final concentration of 2 μmol/ml (1.2 mg/ml total lipid).

Adenovirus

AdCMVNLSβgal is an E1a/E3-deleted Ad5-based adenoviral vector containing the β-galactosidase gene under control of the CMV early promoter and enhancer. AdCMVNLSβgal infectious particles were prepared by standard methods as previously described.[12]

Cell transfection using cationic liposomes

Cells were plated in 24-well tissue-culture grade plates (Greiner Labortechnik) at 1×10^5 per well and maintained at 37°C/5% CO_2 until 60–70% confluent in their respective growth media. Cells were washed with PBS (pH 7.4) and the medium replaced with either 400 μl of OptiMEM (positive control) or the equivalent volume of sputum diluted in OptiMEM at the ratios described above. The DNA and liposomes were complexed by mixing of the two components at a ratio of 1:5 weight/weight DNA:liposome, and immediately added to the cells (5 μg DNA per well). Media or media/sputum mixtures were removed 18–24 h after transfection, the cells were washed twice with PBS and the media replaced with the appropriate normal growth media.

Cell transfection using adenovirus

Cells were grown as described above. Before transfection, cells were washed with PBS and 400 μl of MEM or the equivalent volume of sputum diluted in MEM at the ratios described above were added. AdCMVNLSβgal (10 μl containing 6.5×10^5 p.f.u./μl) was added to each well to give an estimated MOI of 6.5. Media or media/sputum mixtures were removed after 2 h and replaced with the appropriate normal growth media for each cell line.

β-Galactosidase assay

The cells were assayed for β-galactosidase activity using a photometric assay previously described.[35] Briefly, 36 or 48 h after transfection using cationic liposomes or adenovirus respectively, the cells were lysed in a solution containing 0.1% Triton X-100 and 250 mm Tris (pH 8, 350 μl per well), frozen at −70°C and thawed at room temperature. From each well 50 μl of cell lysate was transferred to a 96-well plate and to each well was added 50 μl PBS containing 0.5% BSA, 150 μl chlorophenol red galactopyranoside (1 mg/ml in a buffer containing 60 mm Na_2HPO_4, 1 mm $MgSO_4$, 10 mm KCl and 50 mM β-mercaptoethanol). Following 60 min incubation at 37°C, the plate was read at 578 nm in a microtitre plate. Absorption readings were converted to pg of β-galactosidase using a standard curve of β-galactosidase. To minimize the effects of differing cell numbers and cell growth, the amount of β-galactosidase expression obtained was standardized by calculation of the amount of protein present in each cell lysate. Protein was assayed using the Bradford method[36] in a microassay protocol adapted for use in 96-well plates (Bio-Rad, Hemel Hempstead, UK) read at 595 nm. Absorption readings were converted to μg protein using a bovine serum albumin standard curve. Thus data are expressed as pg β-galactosidase/μg protein.

Removal of sputum with PBS and with mucolytic agents

Cells were covered with sputum (diluted 1:10 sputum:OptiMEM) and incubated at 37°C/5%CO_2 for 60 min. Sputum was then removed from each well by washing three times with PBS. This was followed by the addition of 400 μl OptiMEM per well and the transfection procedure followed as described above. Alternatively, following washing with PBS, the cells were incubated for a further 60 min in the presence of one of the following mucolytic agents *N*-acetylcysteine, lysine (both Sigma, Poole, Dorset, UK), Nacystelyn (Galephar SMB, Brussels, Belgium) (each 20 mm, pH 7.4), recombinant bacterial alginate lyase (0.5 enzyme units per well; a gift from P Gacesa, Department of Applied Biology, University of Central Lancashire, UK) and rhDNase (50 μg/ml) (Pulmozyme; Genentech, Welwyn Garden City, UK). Of these agents, only *N*-acetylcysteine and rhDNase are in clinical use. In the case of the former the concentration chosen was the highest shown not to be cytotoxic to epithelial cells in culture.[37] In the case of the latter, the concentration chosen reflects the concentration used *in vivo*, based on the daily nebulised dose of 2.5 mg reaching the conducting airways with a surface area of approximately 60 cm^2.[38] All the agents were removed by washing with PBS, replaced with OptiMEM and the cells transfected as described above.

Determination of the effect of rhDNase on the functional efficiency of the gene–liposome complex

Cos 7 cells were plated as described above. The transfection procedure was similarly carried out as above, but in the presence of increasing concentrations of rhDNase, added to each well at the same time as the addition of the DNA–liposome complexes. Thus, the following amounts of rhDNase were added: 0.5 μg/ml, 5 μg/ml, 50 μg/ml, 100 μg/ml and 500 μg/ml.

Preparation of genomic DNA from rat lung

As previously described,[39] 1.5 g of rat lung were incubated in 9 ml proteinase K buffer (100 mm NaCl, 100 mm EDTA, 50 mm Tris-HCl, pH 8, 1% SDS, 6 mg/ml proteinase K (Gibco BRL, Life Technologies, Paisley, UK)) overnight at 55°C. Following two extractions with phenol: chloroform:isoamyl alcohol (25:24:1, Gibco BRL, Life

Technologies) the DNA was recovered through ethanol precipitation. The DNA pellet was washed in 70% ethanol and resuspended in sterile water.

Determination of the effect of genomic DNA on gene transfer efficiency in vitro

Cos 7 cells were incubated at 37°C/5%CO_2 for 1 h with genomic DNA diluted to the following concentrations with OptiMEM: 1 μg/ml, 10 μg/ml, 100 μg/ml and 500 μg/ml. The DNA was removed by washing with PBS and the cells incubated for a further 60 min in the presence of rhDNase (50 μg/ml). The latter was removed by washing with PBS, replaced with OptiMEM and the cells transfected as described above.

Statistics

Samples were studied in triplicate and the mean used for further analysis. The data are presented as mean ± s.e.m. for convenience, where *n* refers to numbers of patients. The Mann–Whitney *U* test was used for comparisons between interventions and the null hypothesis rejected at $P < 0.05$.

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