



NONVIRAL TRANSFER TECHNOLOGY

RESEARCH ARTICLE

Cationic lipid-mediated gene transfer to the growing murine and human airway

A Jaffé^{1,2}, D Judd¹, C Ratcliffe³, SH Cheng⁴, A Bush², DM Geddes¹ and EFW Alton¹

¹Department of Gene Therapy, Imperial College School of Medicine and Technology at the National Heart and Lung Institute, London; ²Department of Respiratory Paediatrics; ³Department of Thoracic Surgery, Royal Brompton and Harefield NHS Trust, London, UK; and ⁴Genzyme Corporation, Framingham, MA, USA

Gene therapy in patients with cystic fibrosis may need to be commenced before the onset of lung disease which may be evident as early as 4 weeks after birth. We assessed the efficacy of cationic lipid-mediated transfer of a reporter gene, chloramphenicol acetyltransferase, in the growing murine and human respiratory tract. Gene expression was greater in adult mice (greater than 8 weeks old) compared with 9- and 16-day-old animals, despite a relatively greater proportion of complex delivered to the younger mice. Subsequent experiments compared 16-day-old and adult mice. Whilst higher gene expression occurred in the parenchyma compared with conducting airways in both groups, significantly greater expression was seen in the conducting airway

of adult mice compared with 16-day-old animals. This expression persisted beyond 18 days in the adults but was undetectable in the younger group at this time-point. In an *ex vivo* model there was no difference in gene expression between the two groups. Further, no differences were observed in gene expression between growing (age 5 weeks to 14 years 8 months) and adult human lung tissue in either parenchyma or conducting airway. These data suggest age-dependent differences in gene transfer *in vivo*, which are not seen in an *ex vivo* setting. Proof-of-principle has been demonstrated for cationic-lipid mediated gene transfer to the growing human lung. Gene Therapy (2000) 7, 273–278.

Keywords: growing lung; gene therapy; liposome

Introduction

The human baby born with cystic fibrosis (CF) has predominantly normal lungs at birth¹ but there is evidence of lung inflammation as early as 4 weeks.² Since the cloning of the gene for CF in 1989,³ there has been much interest in the development of gene therapy. This has now reached the stage of clinical trials in adults⁴ but these patients already have established disease. It would be reasonable to hope that future treatments could be commenced in infants before the onset of lung damage.

Viral-based reporter gene studies in animal models suggest that gene expression is comparable, or even greater, in the growing than in the mature airway both in magnitude and duration of expression.^{5–8} Because viral vectors induce an inflammatory response⁹ this increase in gene transfer efficacy in the neonatal animal is partly explained by immunological naivety.^{5,7} To the best of our knowledge, cationic lipid-mediated gene transfer has not been studied in the growing mammalian airway. This is relevant since these gene transfer agents offer a number of potential advantages over viruses for human gene therapy. Several groups have reported successful *in vivo* gene transfer to the murine^{10,11} and human^{12–16} airway

following topical administration of cationic lipid–DNA complexes.

In primary cultures of human ciliated airway cells increased transgene expression is related to the rate of cell division following cationic lipid-mediated gene transfer.¹⁷ If this holds true *in vivo*, one would expect higher transfection rates in the growing airway due to the increased level of cell mitosis compared with adults.¹⁸

The aim of this study was to assess the magnitude of expression, duration and site of cationic lipid-mediated gene transfer to the growing murine airway and to extend this to the growing human airway.

Results

Effect of age on gene transfer

Following *in vivo* gene transfer, transgene expression in whole lung homogenates (standardised to body weight at instillation) increased with increasing age of mice from day 16 onwards (Figure 1). No significant differences were seen between day 9 and 16-day-old animals, but thereafter there was a significant ($P < 0.05$) increase in gene expression compared with 16-day-old mice. Standardising gene expression for lung weight or lung homogenate protein content (data not shown) did not alter these findings.

Site of gene expression

Gene expression, assessed 48 h after delivery, was greater in parenchymal tissue than in the conducting airway in

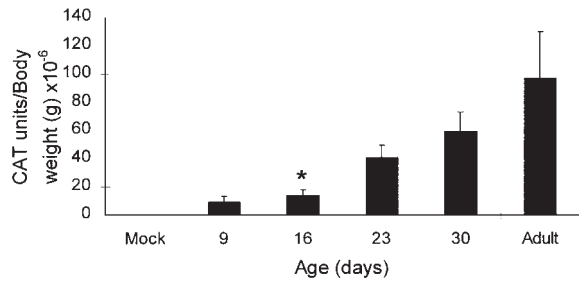


Figure 1 Effect of age on gene expression. CAT expression standardised for body weight at instillation. * $P < 0.05$ comparing 16-day-old animals with older mice ($n = 6-12$). Error bars indicate s.e.m.

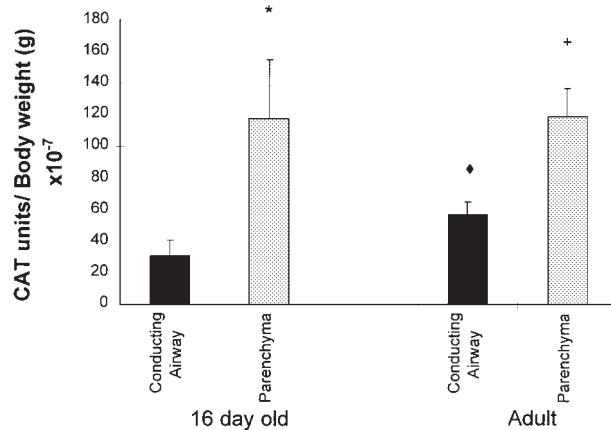


Figure 2 Site of gene expression 48 h following administration. *Indicates $P = 0.05$ for 16-day-old parenchyma versus 16-day-old conducting airways ($n = 6$). *Indicates $P < 0.01$ for adult parenchyma versus adult conducting airways ($n = 6$). ♦Indicates $P < 0.05$ for adult versus 16-day-old conducting airway. Error bars indicate s.e.m.

16-day-old ($P = 0.05$) and adult mice ($P < 0.01$) (Figure 2). There was no significant difference in expression in the parenchymal tissue between the two groups. However, significantly ($P < 0.05$) greater CAT expression was seen in the adult conducting airway compared with the 16-day-old airway.

Assessment of distribution of complex

Because higher levels of gene expression were noted in the older mice, the relative deposition of complexes was assessed in 16-day-old and adult animals (Figure 3). A significantly greater amount of complex reached both the parenchyma ($P < 0.001$) and the conducting airway ($P < 0.05$) in the 16-day-old animals. In total, $7.2\% \pm 0.2$ of the instilled solution reached the lungs in 16-day-old mice compared with $4.8\% \pm 0.4$ in adults ($P < 0.001$). Thus, the higher expression levels seen in the adult animals are not due to increased complex deposition. In both groups of animals there was a significantly greater deposition in the parenchyma compared with the conducting airways as would be expected using this means of delivery.

Duration of gene expression

We compared the duration of expression in 16-day-old and adult mice. Expression was maximal 4 days after gene transfer in both groups (Figure 4a and b). By 18 days

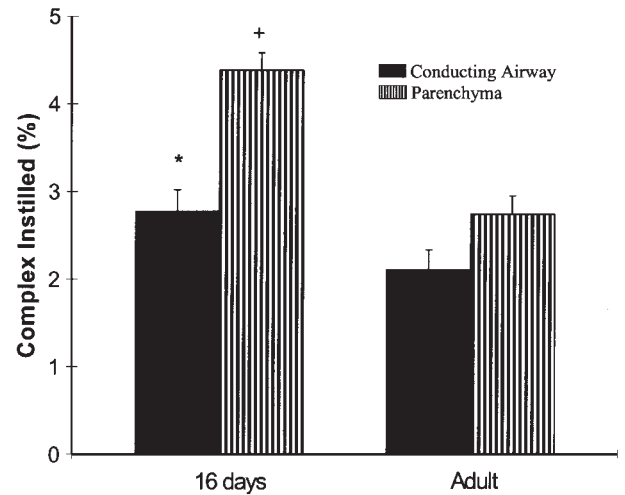
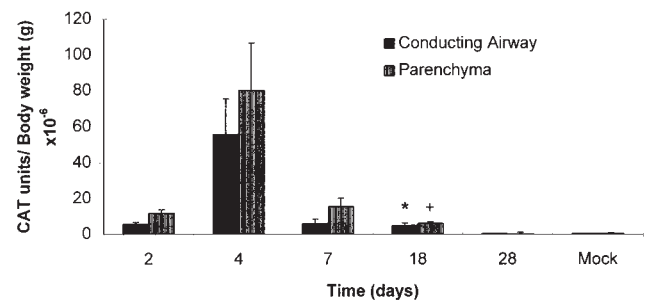


Figure 3 Percentage of instilled complex reaching the lower airways. * $P < 0.05$ and + $P < 0.0001$ for conducting airway and parenchyma, respectively, when comparing 16 day ($n = 9$) and adult animals ($n = 12$). For both groups of animals parenchymal distribution was significantly greater than in conducting airways (16 day $P < 0.001$, adult $P < 0.05$). Error bars indicate s.e.m.

a Adult mice



b 16 day mice

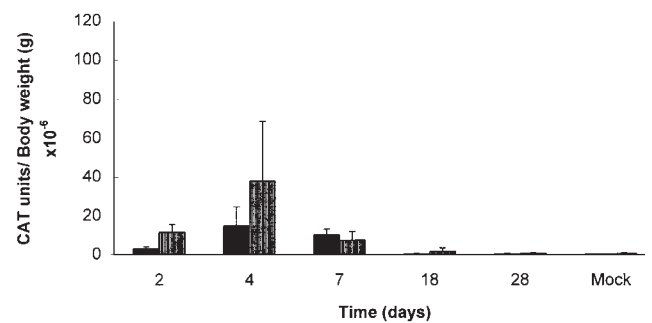


Figure 4 Duration of gene expression in (a) adult and (b) 16 day-old mice ($n = 6$ each group at each time-point). * $P < 0.05$ comparing adult and 16-day-old conducting airways. + $P < 0.01$ comparing adult and 16-day-old parenchyma. Error bars indicate s.e.m.

gene expression was undetectable in the young mice but was still present in the adults (conducting airway $P < 0.05$, parenchyma $P < 0.01$ compared with 16-day-old animals). By day 28 expression in the adult group was also undetectable. At nearly all time-points gene expression was greater in the parenchyma than conducting airway for both groups.

Gene expression in the 16-day-old and adult murine airway assessed *ex vivo*

There was no difference in gene expression between 16-day-old and adult murine tissues, with respect to either the trachea or parenchyma following transfection in an *ex vivo* model system (Figure 5). As for the *in vivo* studies, expression was greater in the parenchyma than the trachea in both age groups, although this did not reach significance (16 day old $P = 0.53$, adult $P = 0.20$).

Gene expression in the growing and adult human airway assessed *ex vivo*

Significant gene expression was demonstrable in both the adult conducting airway ($P < 0.001$) and parenchymal tissue ($P < 0.01$) compared with untransfected tissues (Figure 6a). Similarly, significant gene expression could be detected in the paediatric lung tissues in the conducting airways ($P < 0.05$) and parenchyma ($P < 0.01$) (Figure 6b). As for the murine tissues there was no significant difference in gene expression between the growing and adult conducting airways or parenchyma.

Discussion

These studies have demonstrated for the first time successful cationic lipid-mediated gene transfer to the growing native murine and human lung *ex vivo*. The results suggest that gene expression is greater and lasts longer in adult mice compared with younger mice *in vivo*. In both groups higher gene expression is seen in the parenchyma (fourth generation bronchi onwards) compared with the conducting airway. However, in both murine and human *ex vivo* studies no difference in gene expression was found between the adult and immature lung.

The various ages chosen to assess gene transfer were based on the known developmental stages of the growth of the mouse lung. At birth while the tracheobronchial tree is formed as in man,^{18,19} murine alveoli exist only as respiratory sacculles. Rapid alveolarisation commences on days 3–4 after birth and is completed by day 14 when an appearance similar to the adult is achieved.²⁰ This is equivalent to the human lung at 2–4 years. The most rapid increase in lung weight in the mouse is in the first 10 days and the lung continues to grow rapidly until 8

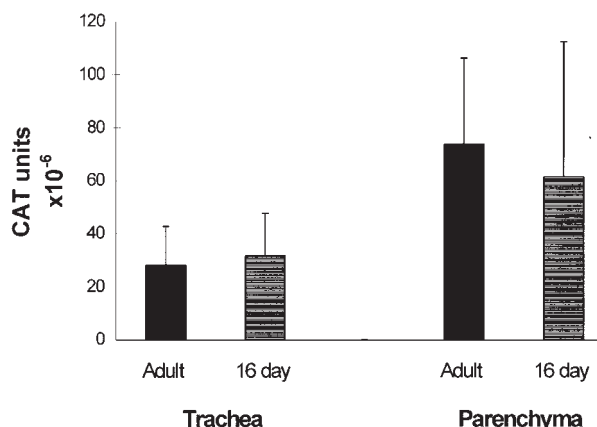
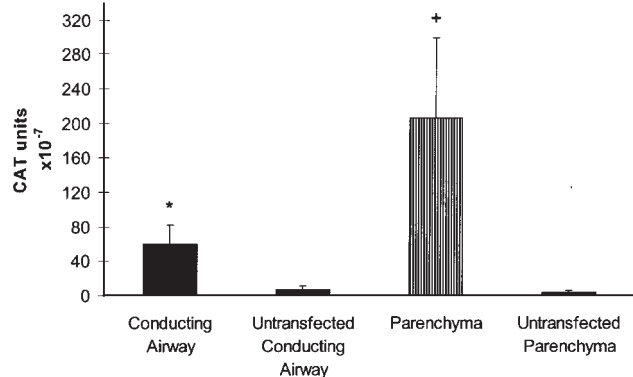


Figure 5 Gene expression in the trachea and parenchyma of adult ($n = 6$) and 16-day-old mice ($n = 6$) assessed *ex vivo*. Error bars indicate s.e.m.

a Human adult airway



b Human growing airway

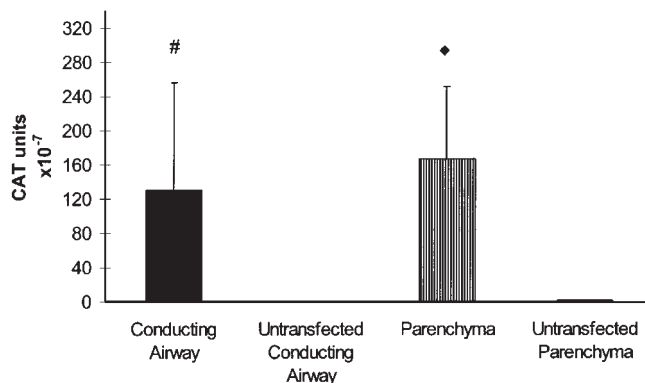


Figure 6 Gene expression in human (a) adult and (b) growing conducting airways and parenchyma. * $P < 0.001$ adult conducting airway ($n = 8$) and $P < 0.01$ adult parenchyma ($n = 8$) compared with untransfected tissue. # $P < 0.05$ growing conducting airway ($n = 6$) and ♦ $P < 0.01$ growing parenchyma ($n = 8$) compared with untransfected tissue. Error bars indicate s.e.m.

weeks of age when growth slows down.^{20,21} The relationship of lung to body weight growth is linear.²⁰ Since significant changes in gene expression were seen after day 16 compared with the older mice (Figure 1) it was decided to use the 16-day-old and adult groups for future studies. Further, the 16-day-old mice resembled the older age groups in both appearance and response to anaesthesia more closely than the 9-day-old mice.

We initially hypothesised that the difference in gene expression in murine whole lung homogenates was due to more efficient delivery of the DNA–liposome complexes to the lungs in the older animal. However, more complex was delivered to the younger mice. Interestingly, only 4–7% of the total complex was recovered from the lungs following intranasal inhalation. It is probable that most of the complex is retained in the nose or swallowed.

Higher levels of gene expression were seen in the parenchyma than in conducting airways in both the 16-day-old and adult mice both *in vivo* (Figures 2 and 4) and *ex vivo* (Figure 5) in keeping with previous studies in adult animals showing preferential transfection of alveolar cells.^{11,22} *In vivo*, this is likely to be due to greater delivery of the complex to the parenchyma compared with conducting airway (Figure 3). *Ex vivo* this may represent preferential transfection of alveolar or other cells not present in conducting airway. It is also feasible that the

CMV promoter is more active in alveolar cells rather than surface epithelial cells.

We suggest that the difference in gene expression seen in 16-day-old and adult whole lung homogenates is due to the significant differences in expression in the conducting airway (Figure 2). Although we standardised DNA dose for body weight, lung weight or lung protein we were unable to do so for surface area, likely the most important parameter. In their study of the ferret airway, Oldham *et al*²³ suggested that the surface airway of conducting airway of mammals is proportional to the crown-rump length of the animals. Analysis of our data, taking into account differences in surface area of conducting airway by this method, did not alter the significance in gene expression between younger and older mice. We do not know the reason for the differences between our *in vivo* and *ex vivo* findings, but a number of factors such as differences in ciliary clearance or increased macrophage scavenging in the growing lung need to be considered.

We studied the duration of gene expression in 16-day-old and adult mice. Our adult data are similar to Lee *et al*²² who demonstrated a peak in transgene expression between 1 and 4 days after instillation in adult female BALB/c mice, with persistence to 21 days. No expression was demonstrable in the 16-day-old mice 18 days after gene delivery (Figure 4a and b). This is in direct contrast to previously published gene transfer studies using viral vectors in which gene expression was significantly longer in younger animals.⁵⁻⁷ One explanation may be the marked changes occurring in the developing lung during the alveolar phase, with significant remodelling of the epithelium. Rubinstein *et al*²⁴ have suggested that this period, as well as the period following the alveolar phase, is associated with significant cell proliferation and differentiation from stem cells. They suggest that successful gene transfer of the airway epithelial cells may be 'diluted' if the vector is not propagated during cell mitosis or if the pulmonary epithelia are derived from 'new' stem cells. This may provide an explanation since 16-day-old mice are just completing the alveolar phase of development and liposome-mediated gene transfer results in episomal maintenance of the transgene.

Gene expression in the growing human conducting airway and parenchymal tissue is as efficient as that seen in the adult *ex vivo*. Although it is difficult to extrapolate these results to the airway of children with CF, two of our paediatric patients had bronchiectasis, but still demonstrated gene expression.

In conclusion, we have demonstrated for the first time that cationic lipid-mediated gene transfer is possible to the growing murine and human airways. Our data suggest that gene transfer is more efficient in adult mice *in vivo*. However, these differences were not borne out in the human or murine lung tissue *ex vivo*. It is likely that the paediatric CF lung will be a target for gene therapy. These results support the use of cationic lipid vectors in gene therapy trials involving the growing airways and may have implications in the design of future gene therapy trials in children.

Materials and methods

Lipid preparation

The cationic lipid, GL-67, was complexed with dioleolyphosphatidylethanolamine (DOPE) at a molar ratio of 1:2

as described previously²² and stored at -70°C as a dry powder. Sterile endotoxin-free water was used to rehydrate the lipid to a concentration of 1.2 mM before use.

Plasmid preparation

The plasmid pCF1-CAT construct containing the human CMV immediate-early promoter and enhancer, a hybrid intron, the bovine growth hormone polyadenylation signal sequence, a pUC origin of replication, the kanamycin resistance gene and bacterial DNA coding for chloramphenicol acetyltransferase was prepared using Qiagen Mega Kits (Crawley, Sussex, UK). pCF1- β Gal was constructed in a similar way except that the cDNA for chloramphenicol acetyltransferase was replaced by β -galactosidase. Endotoxin was removed using the Qiagen Endofree Plasmid Maxi Kits. The plasmid was resuspended in endotoxin-free water and DNA integrity checked by restriction digests. The concentration of DNA was adjusted to 1.6 mg/ml (4.8 mM) using a Unicam UV/VIS spectrometer at 260 nm (Unicam Instruments, Cambridge, UK).

Complex preparation

Both DNA and lipid (mM ratio 4:1, DNA:lipid) were incubated at 30°C for 5 min before lipid was added to DNA. The solution was incubated for a further 15 min before instillation. In studies to assess the site of distribution of complex, 20 μl of tritiated thymidine ($^3\text{HTdr}$; Amersham Life Sciences, Buckinghamshire, UK) was added to the lipid before the addition of DNA.

Animals

Female BALB/c mice (Harlan, Oxfordshire, UK) were housed in a temperature-controlled (21°C) room. Food (Special Diet Services, Witham, UK) and water were freely available. Mice were considered adult after 8 weeks of age.²¹

Intranasal instillation

Mice aged 16 days and above were anaesthetised using metaphane (Mallincrodt Veterinary, Mundelein, IL, USA) and instilled intranasally with 4 μl of lipid:DNA complex (3.2 μg DNA)/g body weight as described previously.²² Briefly, the jaw was immobilised closed and the droplets were applied to the nose using a Gilson Pippetman (Anachem, Bedfordshire, UK) and the complex sniffed into the lung. No anaesthesia was used in the 9-day-old mice.

In vivo protocols

Effect of age on gene expression: CAT expression was measured in whole lung homogenates at 48 h following gene transfer in mice aged 9, 16, 23, 30 days and adult ($n = 6-12$ each group).

Assessment of site of gene expression: Lungs were removed 48 h after delivery and separated into 'conducting airways' (trachea to third branching generation) and parenchymal tissue by blunt dissection and then weighed ($n = 6$ each group).

Assessment of age on distribution of complex: To evaluate the effect of age on delivery of the lipid-DNA complex to the lung by nasal inhalation, levels of tritiated

thymidine were measured in the conducting airway and parenchyma in mice aged 16 days ($n=9$) and adults ($n=12$) immediately following inhalation of 4 μl of tritiated complex/g body weight.

Assessment of duration of expression: CAT expression was assessed in the parenchyma and conducting airways at days 2, 4, 7, 18 and 28 following instillation in adult and 16-day-old mice ($n=6$ each group at each time-point).

Preparation of cell lysate

Tissues were homogenised for 10 s in 500 μl Tris-HCl (250 mM pH 8.0, Sigma T3038) at 24000 RPM in an Ultra Turax T25. Cells were lysed by four freeze-thaw cycles (5 min at -80°C and 37°C) with the final cycle heated at 60°C for 10 min to inactivate mammalian deacetylases.

CAT assay

CAT activity was assayed as described previously.²⁵ A microcentrifuge containing 100 μl Tris-HCl (pH 8.0, 250 mM), 20 μl of cell lysate, 5 μl of n-butyryl CoA and 5 μl of D-threo (dichloroacetyl- $1\text{-}^{14}\text{C}$) chloramphenicol (Amersham 1.85 MBq/ml) was incubated at 37°C for 90 min. Each reaction was terminated using 300 μl of xylene. Each sample was vortexed for 10 s and spun at 16 000 g_{av} at 4°C for 6 min. The supernatant was removed and washed with 100 μl Tris-HCl (250 mM, pH 8.0), vortexed and spun as before. This was repeated twice. Two hundred μl of the final supernatant was added to 3 ml of Optifluor (Canberra Packard, Berkshire, UK) and the disintegrations per minute (d.p.m.) counted for 1 min using a Canberra Packard 2500 TR liquid scintillation analyser. Results are expressed as CAT units/g body weight at instillation \pm s.e.m. in the *in vivo* work, and as CAT units \pm s.e.m. in the *ex vivo* studies.

Protein assay

The protein content of each sample was determined using the BioRad assay dye reagent concentrate (BioRad Laboratories, Hertfordshire, UK). Protein concentrations of the cell lysates were determined by calculating values from a standard curve constructed using bovine serum albumin.

Tritiated thymidine assay

After recovery from anaesthesia, mice were immediately killed and the lungs removed and divided into parenchyma and CA by blunt dissection. Cell lysates were prepared and the final lysate was added to 3 ml of Optifluor and d.p.m. counted as described above. An indication of the concentration of $^3\text{HTdr}$ in the instilled complex was assessed by counting the d.p.m. in 10 μl of the instilled lipid-DNA complex. Results are expressed as percentage of total complex instilled \pm s.e.m.

Ex vivo model of gene transfer

Adult mice ($n=6$) and mice aged 16 days ($n=6$) were killed following intraperitoneal anaesthesia (Avertin, Sigma, Poole, UK) and the lung bloc removed. Human parenchyma and conducting airway were obtained from paediatric ($n=8$) and adult ($n=8$) patients undergoing lobectomy or pneumonectomy. Patient characteristics are summarised in Table 1. The study was approved by the Ethics Committee of the Royal Brompton Hospital.

Table 1 Patient characteristics

Age	Diagnosis
<i>(A) Adult</i>	
31 years	Bronchiectasis
50 years	Squamous cell carcinoma
50 years	Non small cell carcinoma
62 years	Non small cell carcinoma
65 years	Adenocarcinoma
66 years	Adenocarcinoma
72 years	Non small cell carcinoma
75 years	Non small cell carcinoma
<i>(B) Paediatric</i>	
5 weeks	Congenital lobar emphysema
6 months	Cystic adenomatoid malformation
10 months	Congenital lung emphysema
4 years 2 months	Bronchogenic cyst
5 years 9 months	Bronchiectasis
6 years 4 months	Cystic adenomatoid malformation
13 years 10 months	Osteosarcoma secondaries
14 years 8 months	Bronchiectasis

Lung tissue (murine or human) was transported to the laboratory immersed in Krebs-Henseleit Solution (KHS) of concentration (mm): Na^+ 145, Cl^- 126, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 26, PO_4^{2-} 1.2, SO_4^{2-} 1.2, glucose 5.6, pH 7.2 when gassed with 95% O_2 /5% CO_2 . The tissue was divided into sections approximately 3 mm^2 and laid on an air-liquid interface adapted from previously described methods.²⁶ This consisted of a 35-mm Petri dish aseptically placed within a 55-mm Petri dish with a filter paper (Whatman No. 1, Maidstone, UK) laid across the inner dish with either end reaching the outer container. Four ml of minimal essential media (MEM/A, Sigma) containing 50 $\mu\text{g}/\text{ml}$ gentamicin and 1 \times antibiotic/antimycotic solution (5 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 250 ng/ml amphotericin B) was placed in the outer dish allowing the filter paper to soak up this solution. Each piece of tissue was placed in the centre of the filter paper in the inner dish with the mucosal surface facing upwards (conducting airway). In the human experiments an 'O' ring of diameter 0.31 cm was placed in the centre of the tissue and secured with liquefied agar. A 5 μl aliquot of plasmid-DNA complex was applied to the centre of the murine tissue or to the inside of the 'O' ring in the human experiments and incubated for 48 h at 37°C /5% CO_2 . In the murine and human parenchymal tissue the complex was applied to the exposed cut surface. Untransfected tissue was used as a control. Cell lysates were prepared as described above. The viability of the conducting airway tissue was confirmed by the presence of beating cilia at the beginning and at the end of the experiment by light microscopy. Because of the large differences in the amount of non-epithelial protein present in the 16 day compared with the adult mouse trachea, CAT expression could not be standardised in this way. As indicated above, tissues of approximately equal surface area were therefore used. The number of cells/ μm of trachea did not differ between 16-day-old (0.217 cells/ $\mu\text{m} \pm 0.004$, $n=6$) and adult trachea (0.238 cells/ $\mu\text{m} \pm 0.010$, $n=6$) assessed using an image analysis system.

Chemicals

All chemicals were from Sigma unless otherwise stated and were of AnalaR grade or best available.

Statistics

The Mann–Whitney *U* test was used to compare groups and the null hypothesis rejected at $P < 0.05$. Values are expressed as mean \pm s.e.m. for convenience.

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