Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses

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We describe the use of a human bronchial xenograft model for studying the efficiency and biology of *in vivo* gene transfer into human bronchial epithelia with recombinant E1 deleted adenoviruses. All cell types in the surface epithelium except basal cells efficiently expressed the adenoviral transduced recombinant genes, *lacZ* and CFTR, for 3–5 weeks. Stable transgene expression was associated with high level expression of the early adenoviral gene, E2a, in a subset of transgene expressing cells and virtually undetectable expression of the late adenoviral genes encoding the structural proteins, hexon and fiber. These studies begin to address important issues that relate to safety and *in vivo* efficacy of recombinant adenoviruses for gene delivery into the human airway.

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Cystic fibrosis (CF) is an autosomal recessive disease that is associated with pathology in multiple organ systems. The pulmonary complications in CF, which are believed to be caused by defects in mucociliary clearance, are the most morbid aspects of the disease and the most difficult to treat[1]. Isolation of the gene responsible for CF, and the characterization of its protein product — cystic fibrosis transmembrane conductance regulator (CFTR) — suggested possible therapeutic strategies based on lung directed somatic gene transfer[2–6]. Correction of the functional defect in CF cells by viral mediated transduction of a normal CFTR gene further supported the feasibility of gene therapy for CF[7,8].

Rational development of therapies for CF pulmonary disease based on somatic gene transfer requires a consideration of the possible target cells in the lung. Extensive clinical experience has suggested that the primary abnormalities in the CF lung reside in the conducting airways[1]. Several observations suggest that the surface epithelial cells of the airway play a direct role in pathogenesis of CF. The functional abnormality characteristic of CF (that is, defective regulation of Cl conductance) has been demonstrated in excised CF nasal epithelia[9] and in epithelial cells from proximal airways of CF patients[10]. CFTR RNA and protein have also been detected in surface epithelial cells from the proximal and distal airway of non-CF patients[11–13]. One hypothesis to explain pathogenesis in the lung is that CFTR–mediated anion transport, and associated Na and water transport, directly affect the hydration and corresponding rheological properties of mucus in the airway[14]. CFTR is also expressed at high levels in submucosal glands of the proximal human airway suggesting that these structures may also play a role in primary pathogenesis[15]. The relative contributions of airway surface epithelia and submucosal glands to the pathogenesis of CF lung disease are unclear.

Initial strategies for gene therapy of CF lung disease have focused on the surface epithelium of the airway as the target for CFTR gene transfer. One significant advantage of this approach is that surface epithelial cells can be exposed to the CFTR gene *in vivo* using noninvasive and clinically practical approaches such as lavage or inhalation. While this is an important advantage, the surface epithelium offers unique challenges to the development of effective gene therapies. The surface of the human proximal airway is a complex pseudostratified epithelium containing multiple cell types including ciliated cells, secretory cells, basal cells, and intermediate cells[16]. The lineage relationships within this epithelium are also complex with the basal and secretory cells functioning as potential progenitors[17]. Finally, under normal conditions the surface epithelium has a low proliferative rate[18], making it a difficult target for gene transfer using techniques that depend on transgene integration for stable expression such as retroviruses[19].

In order to study the feasibility of direct gene transfer into the human airway, we have developed an animal model that is based on the growth of human airway epithelial xenografts in immunodeficient mice. This model was used to study the efficiency and consequences of *in vivo* gene transfer via recombinant adenoviruses.

Characterization of xenograft epithelium

Xenografts seeded with 1×10^6 freshly isolated human bronchial epithelial cells gave rise to fully differentiated

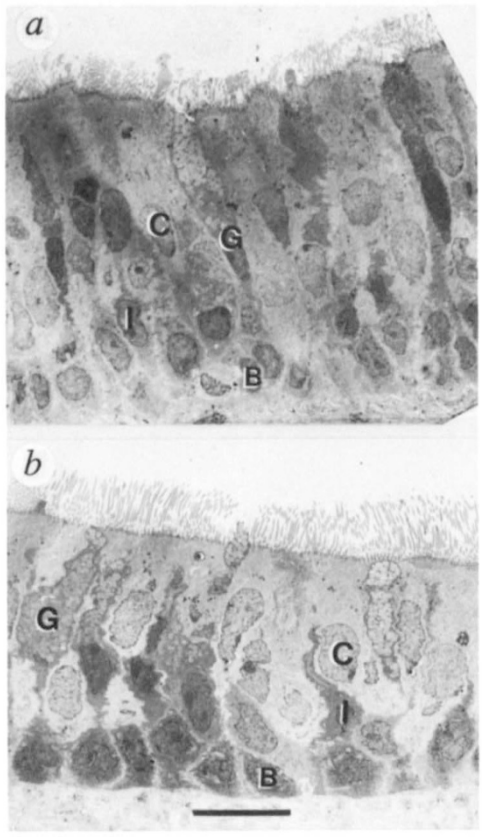
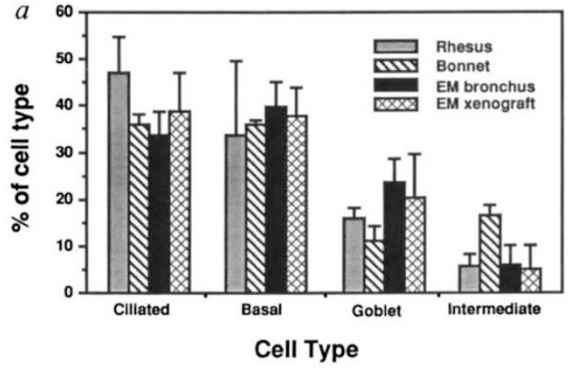
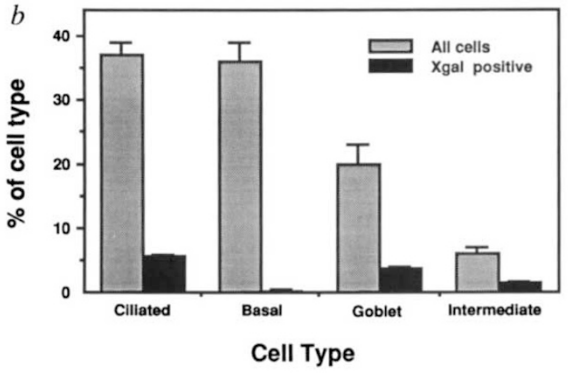


Fig. 1 Electron micrographs of bronchial epithelia from human bronchus and a xenograft. Micrograph of human bronchial epithelium *a*, and epithelium from a xenograft seeded with human bronchial epithelial cells and harvested at 42 days *b*. C, ciliated cell; G, goblet cell; B, basal cell and I, intermediate cell. Scalebar, 20 μm.





epithelia within 3 weeks after implantation into *nu/nu* mice. Transmission electron microscopy demonstrated that the general organization of epithelia in xenografts (Fig. 1*b*) is similar to that found in native airway (Fig. 1*a*). Electron micrographs were analysed morphometrically to evaluate the distribution of cell types found in proximal surface epithelia of these tissues (Fig. 2). The distribution of cell types found in xenografts and bronchial tissues closely resembles that described previously for the proximal airway of primates (Fig. 2*a*)[20,21]. Furthermore, there were no differences in the abundance of ciliated cells, goblet cells, basal cells and intermediate cells noted between the xenografts and bronchial tissue from which the xenografts were derived (see Fig. 2 for statistical analysis). These studies confirm the validity of the xenograft model for studying proximal human airway.

Adenoviral gene transfer in bronchial epithelia

We studied a variety of recombinant adenoviruses based on Ad5. Each virus was deleted of E1 and E3 sequences; some of the recombinants contain a minigene in place of E1. Viruses used in this study include: Ad.E1Δ, the precursor recombinant virus in which E1 and E3 has been deleted without the additional sequences; Ad.RSVβgal, which contains nuclear targeted *lacZ* expressed from a Rous Sarcoma virus LTR; Ad.CMV*lacZ*, which contains cytoplasmic *lacZ* expressed from the CMV promoter; and Ad.CB*CFTR*, which contains human CFTR expressed from the CMV enhancer and β-actin promoter.

Xenografts were infected with purified stocks of Ad.RSVβgal (10^{12}, 10^{9}, and 10^{8} pfu ml^{-1}) or Ad.E1Δ (10^{12} pfu ml^{-1}) for one h after which the virus was expelled. Grafts were harvested, fixed, stained in Xgal and visualized *en face* through a dissecting microscope in order to assess the overall efficiency of infection (Fig. 3). Xenografts infected with Ad.E1Δ demonstrated no Xgal positive cells (Fig. 3*d*, inset), while large areas of *lacZ* expression were demonstrated in grafts exposed to Ad.RSVβgal (10^{12} pfu ml^{-1}) and harvested 3 days later (Fig. 3*d*). Morphometric analysis of GMA sections of this xenograft indicated gene expression in 11 +/-6.3% of the epithelial cells (Fig. 3*e,f*). Similarly high levels of infection were obtained with viral stocks diluted 10–100-fold; infection of 12 xenografts generated from 4 independent tissue samples with 10^{10} to 10^{11} pfu ml^{-1} of Ad.RSVβgal resulted in *lacZ* expression ranging from 5–20% of the cells (data not shown). The inability to achieve an increment in gene transfer at titres of virus greater than 10^{10} pfu ml^{-1} suggest that saturation of the adenoviral receptor has been achieved. Xenografts infected with Ad.RSVβgal at 10^{9} and 10^{8} pfu ml^{-1} and

Fig. 2 Distribution of cell types in several proximal airway samples. *a*, Percentages of ciliated, basal, goblet, and intermediate cells in proximal airways of Rhesus monkeys[20], Bonnet monkeys[21], human bronchus by electron microscopy, human xenograft by electron microscopy. Statistical analysis comparing cell types found in the human bronchus and human xenografts using the students t-test, give the following p-values: ciliated cells, p=0.03; basal cells, p=0.14; goblet cells, p=0.14, intermediate cells, p=0.54. *b*, Percentages of ciliated, basal, goblet, and intermediate cells found in a human bronchial xenograft infected with 10^{12} pfu ml^{-1} Ad.RSVβgal as determined by light microscopy. These values are compared to the distribution of *lacZ* expressing cells in the same xenograft as determined by Xgal staining and light microscopy.

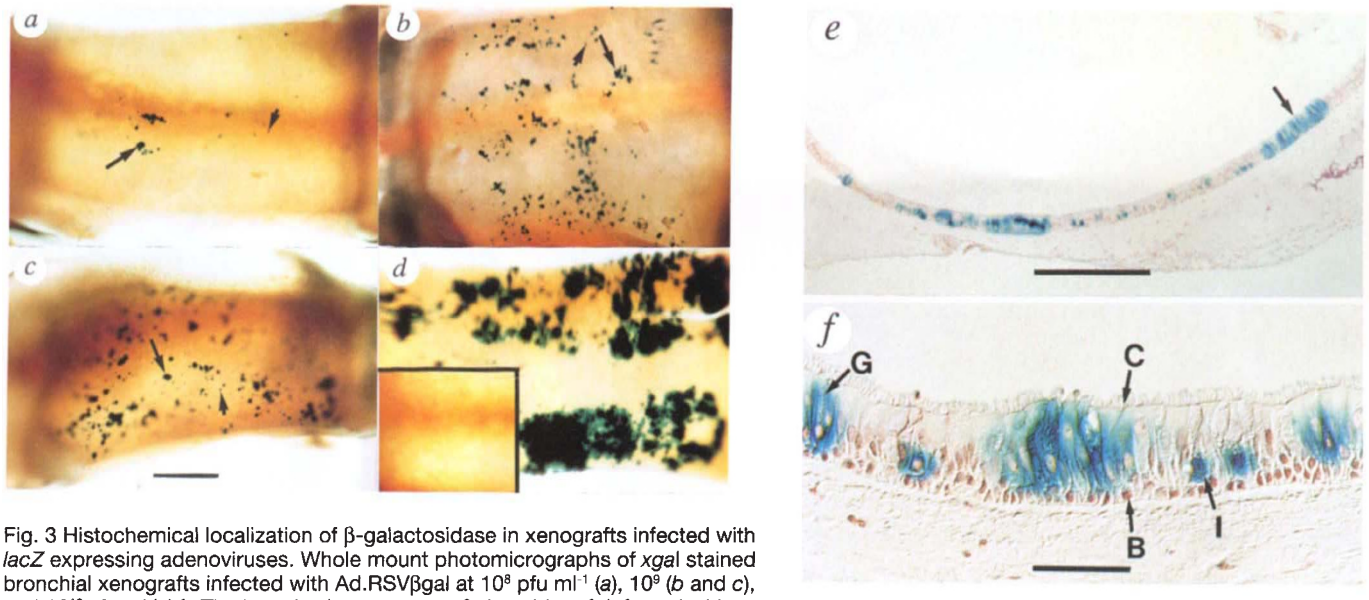


Fig. 3 Histochemical localization of β-galactosidase in xenografts infected with *lacZ* expressing adenoviruses. Whole mount photomicrographs of *xgal* stained bronchial xenografts infected with Ad.RSVβgal at 10^8 pfu ml^{-1} (*a*), 10^9 (*b* and *c*), and 10^{12} pfu ml^{-1} (*d*). The inset in *d* represents a 3 day old graft infected with Ad.E1Δ. All xenografts were harvested at 3 days postinfection except for (*c*) which was harvested 21 days after infusion of virus. Long arrows point to "clonal" aggregates of *lacZ* positive cells while short arrows point to single isolated infected cells (scalebar, 400 μm). GMA sections (4 μm) from Xgal stained xenograft which had been infected with 10^{12} pfu ml^{-1} of Ad.RSVβgal and harvested 3 days postinfection: *e*, scalebar, 300 μm and *f*, scalebar, 50 μm. C, ciliated cell; G, goblet cell; B, basal cell; and I, intermediate cell. Arrow in (*e*) points to an aggregate of *lacZ* positive cells.

examined in 3 days demonstrated gene expression in 1.9 +/-0.2% (Fig. 3*b*) and <0.1% (Fig. 3*a*) of the total cells of the epithelium, respectively. To determine if *lacZ* expression is stable within the bronchial epithelium, xenografts were also harvested 21 days following infection with 10^9 pfu ml^{-1} Ad.RSVβgal adenovirus (Fig. 3*c*). No changes in the percentage of Xgal positive cells were seen between 3 days and 21 days postinfection (compare Fig. 3*b* with 3*c*). Transgene expression is present without diminution in xenografts harvested up to 5 weeks after infection (data not shown).

A series of analyses were performed to determine the distribution of transgene expression in the xenografts. Xgal-stained sections of xenografts infected with 10^{12} pfu ml^{-1} of Ad.RSVβgal were analysed by light microscopy to determine the percentage of each cell type that expressed the transgene (Fig. 2*b*). The distribution of all cell types in the xenograft determined by light microscopy was identical to that established using ultrastructural criteria. The proportion of cells containing Xgal precipitate paralleled the distribution of cell types in the graft with the exception that very few basal cells expressed the transgene. The relative absence of *lacZ* expression in basal cells was demonstrated in grafts infected with either Ad.RSVβgal or Ad.CMV*lacZ* suggesting that transcriptional variation of the viral promoters driving transgene expression is not the cause for the exclusion of β-galactosidase activity found in basal cells.

Diffusion of Xgal precipitate made the quantification of cell types within large, highly expressing clusters difficult (Fig. 3*f*). A more precise definition of the cell types expressing the transgene was achieved by performing immunocytochemistry with antibodies to the reporter gene product β-galactosidase and to cell specific markers of basal cells (cytokeratin 14) and differentiated columnar cells (cytokeratin 18). Figure 4 represents a section of Ad.CMV*lacZ* infected xenograft incubated with antibodies to β-galactosidase (red fluorescence, *b*), cytokeratin 14 (blue fluorescence, *c*), and cytokeratin 18 (green fluorescence, *c*). *LacZ* colocalized with the differentiated cell marker cytokeratin 18 in >99.9% transgene expressing cells ($n = 1500$ cells counted) from both Ad.CMV*lacZ* and Ad.RSV*b*gal infected grafts thereby confirming the observations made from xgal stained grafts.

Further experiments were performed with recombinant adenoviruses expressing human CFTR to establish the validity of this model for developing gene therapies of CF. Xenografts harvested 3 days after infection with recombinant adenoviruses were analyzed for CFTR RNA by *in situ* hybridization and for CFTR protein by immunocytochemistry (Fig. 5). Hybridization above background was detected to the antisense CFTR probe in approximately 2–10% of cells in Ad.CB*CFTR* infected grafts (Fig. 5*a*, see arrow). A similar proportion of cells in these grafts demonstrated overexpression of CFTR protein based on immunocytochemistry with a CFTR specific antibody (Fig. 5*c* and *d*, see arrow). Ad.CMV*lacZ* infected xenografts failed to demonstrate hybridization to the CFTR probe (Fig. 5*b*) or binding to the CFTR antibody (Fig. 5*e* and *f*) that was above endogenous levels. Overexpression of CFTR protein in Ad.CB*CFTR* infected grafts was detected in all differentiated cell types including ciliated cells, goblet cells, and intermediate cells. The recombinant protein localized to the apical surface in most of the ciliated and goblet cells and to the cytosol of intermediate cells. Expression of recombinant CFTR protein was detected in grafts for at least 5 weeks after infection (data not shown).

Expression of adenoviral proteins in xenografts

Immunocytochemical techniques were used to analyse xenografts for expression of adenoviral proteins. Antibodies that recognize hexon, fibre, and the 72 kD E2a

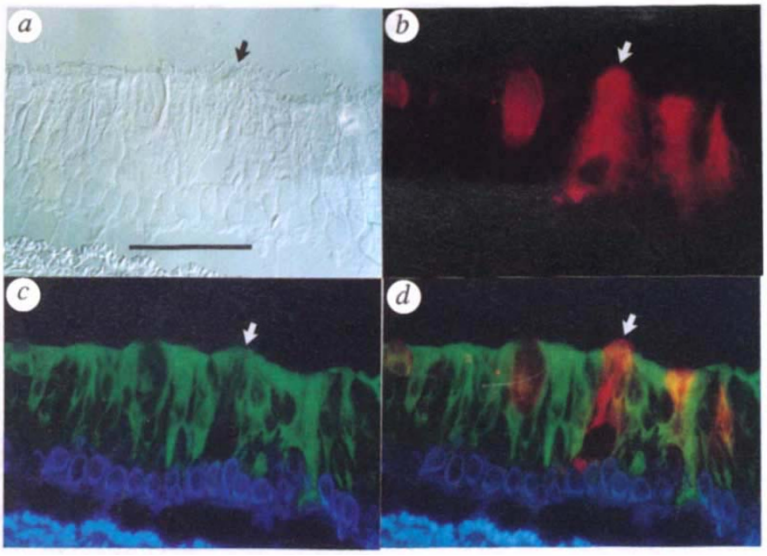


Fig. 4 Cytokeratin expression in β-galactosidase expressing epithelial cells. Frozen sections (6 μm) from xenografts infected with 10^{11} pfu ml^{-1} Ad.CMV*lacZ* and harvested 3 days post infection were analysed by triple immunofluorescence (Nomarski, *a*) with antibodies to β-galactosidase detected with texas red (*b*), cytokeratin 14 detected with AMCA in blue (*c*) and cytokeratin 18 detected with FITC in green (*c*). *d* shows superimposition of all three channels. The arrow in (*d*) indicates a cell that colocalizes β-galactosidase and cytokeratin 18. Scalebar, 25 μm.

gene product [DNA binding protein (DBP)] were used to detect adenoviral protein expression in xenografts infected with Ad.CMV*lacZ*, Ad.RSVβgal, or wild type Ad5. Analysis of xenografts 21 h after infection with wild type Ad5 revealed a subpopulation of cells that expressed high levels of hexon (data not shown) and fibre protein (Fig. 6*f*). Double immunofluorescence studies indicated that cells expressing the late gene products hexon and fiber also expressed low levels of the early gene product DBP (Fig. 6*g*). Similar analyses of xenografts infected with 10^{10}, 10^{11} and 10^{12} pfu ml^{-1} of Ad.RSVβgal (data not shown) or Ad.CMV*lacZ* (Fig. 6*a–d*) failed to demonstrate detectable levels of either fiber or hexon proteins despite substantial levels of β-galactosidase expression. However, with longer incubation times and higher concentrations of primary antibody low levels of fibre expression could be seen in nuclei of a few *lacZ* expressing cells (data not shown). In contrast, high levels of DBP were found in 3–5% of β-galactosidase positive cells with an occasional cell expressing DBP in the absence of detectable β-galactosidase (Fig. 6*a–d*). Cells expressing DBP tended to express lower levels of β-galactosidase and were predominantly found in clusters. The percentage of DBP expressing β-galactosidase positive cells was the same for moi's of 10^{10}, 10^{11} and 10^{12} pfu ml^{-1}.

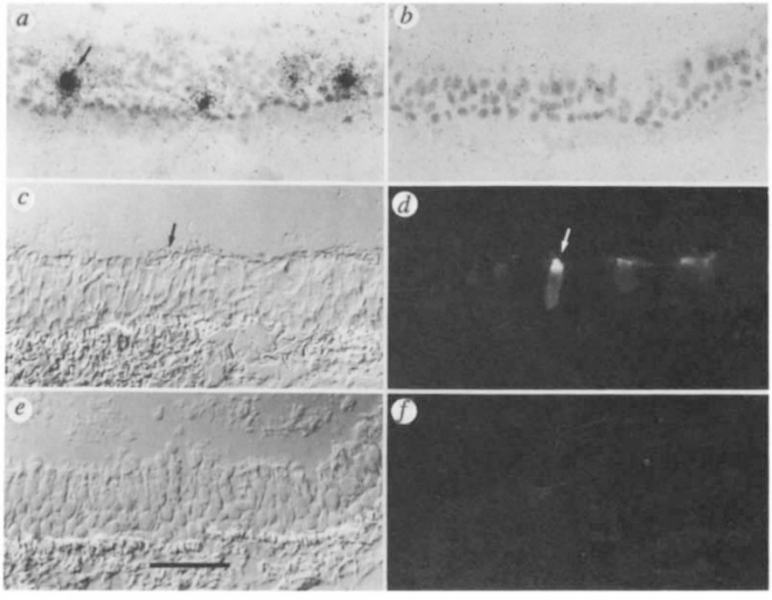


Fig. 5 Localization of transgene derived CFTR in bronchial xenografts. Frozen sections (6 μm) from xenografts infected with either 10^{11} pfu ml^{-1} of Ad.CB*CFTR* or Ad.CMV*lacZ* and harvested 3 days post infection were analyzed by *in situ* hybridization using a human CFTR R-domain probe and by immunocytochemistry for CFTR protein using a polyclonal CFTR antibody[15]. *a*, 1 week exposure of *in situ* analysis on Ad.BA*CFTR* infected xenografts; *b*, 1 week exposure of *in situ* analysis on Ad.CMV*lacZ* infected xenografts. Immunocytochemical analysis for CFTR in xenografts infected with Ad.CBCFTR - Nomarski (*c*) and fluorescence (*d*); and Ad.CMV*laZ* - Nomarski (*e*) and fluorescence (*f*). Arrows indicate cells expressing recombinant CFTR RNA and protein. Scalebar, 50 μm.

Recovery of adenovirus from xenografts

Xenografts were subjected to sequential irrigations for a period up to 24 days after infection. The effluents were evaluated for the presence of recombinant adenovirus using the plaque assay on 293 cells. Fig. 7 presents representative experiments. The concentration of virus in the effluents dropped precipitously during the initial week following infection in all grafts except that presented in Fig. 7*b* in which virus transiently increased before the exponential decline. The amount of virus recovered in the effluents stabilized at low but detectable levels during the remaining period of observation (days 14–24) in most but not all of the grafts. The quantity of virus recovered in effluents at the end of the experiment varied substantially between grafts and was proportional to the percentage of the epithelium that expresses the transgene; the highest concentration of virus was detected in effluents from the xenograft with the greatest level of genetic reconstitution (Fig. 7*a*) whereas the graft that produced effluents with no detectable virus at 24 days was found to have very little transgene expression in its epithelium (Fig. 7*e*). All plaques recovered in the effluents were found to express *lacZ* indicating that the xenografts were not grossly shedding wild type adenovirus. To address further the possibility of wild type adenoviral contamination in the tissue samples or recombinant stocks, 100 μl of selected 1 ml effluents were used to infect 80% confluent layers of Hela cells. Following infection, the media was changed every 48 h for the first 4 days and every day for the following 17 days. No evidence of cytopathic effect was seen with any of the effluents. Finally, PCR analysis of effluents failed to detect E1a sequences at a sensitivity of 100 molecules per ml effluent (data not shown).

Discussion

One approach for treating CF lung disease is to selectively reconstitute CF gene expression in the surface epithelium using gene transfer substrates delivered directly into the airway. Transfection of airway epithelial cells has been

achieved *in vivo* with cationic liposomes, however, the efficiencies have been below what will be required for therapeutic efficacy[22,23]. Efficient and stable recombinant gene expression can be accomplished in proximal airway with recombinant retroviruses if the epithelium is undifferentiated and regenerating at the time of exposure to virus[19], a situation that will be difficult to simulate in patients. An alternative approach is the use of recombinant adenoviruses which have important advantages including (i) they are naturally tropic to the human airway, (ii) they can be grown to extremely high titres, and (iii) transfer and expression of the recombinant gene can be accomplished in nondividing cells[24]. Recombinant adenoviruses have been used to transfer genes for α-1-antitrypsin and CFTR into lungs of cotton rats[25,26].

Despite the tremendous promise of recombinant adenoviruses for lung directed gene therapy, little is known about their biology in the context of the human CF airway. The general strategy is to produce recombinant adenoviruses that are replication defective by virtue of deletions of E1a and E1b sequences. We studied the feasibility of using E1 deleted adenoviruses for CF lung gene therapy by developing an animal model in which human airway epithelial cells from bronchi of NonCF patients were seeded onto denuded rat trachea and implanted into *nu/nu* mice. The xenografts develop a fully differentiated pseudostratified epithelium that is indistinguishable from that found in the endogenous airway. This model has been used to evaluate the feasibility of retroviral mediated gene transfer to the human airway[19]. We have shown that exposure of the xenograft to recombinant adenoviruses results in transgene expression (that is, *lacZ* and human CFTR) in a large number of surface epithelial cells (that is, 5–20% with concentrated virus) and the expression is stable and not associated with pathology. Recombinant gene expression was detected in all cell types of the surface epithelium except basal cells, which may simply relate to abundance of adenoviral receptors on this cell type.

Techniques of immunocytochemistry were used to detect adenoviral proteins in cells of the xenograft that express the recombinant gene[27]. Expression of several adenoviral genes was evaluated including (i) the E2a gene, expressed in the early and late phase of the adenoviral life cycle, that encodes a 72 kD DNA binding protein; and (ii) the L3 and L5 transcripts, formed from the single late transcriptional unit, that encode the structural proteins hexon and fibre, respectively. The program of adenoviral protein expression in cells of the human xenograft differed substantially between wild type Ad5 and the E1 deleted recombinants. Cells infected with wild type Ad5 expressed high levels of hexon and fibre and lower levels of the E2a gene product, indicating they are capable of supporting the full life cycle of Ad5. Cells harbouring E1-deleted Ad5 expressed little if any of the structural proteins whereas *E2a* was expressed at very high levels in a subset of cells. This suggests that the virus is prevented from transitioning into the late phase of transcription in the absence of E1a and E1b. However, a subset of cells is capable of activating transcription from the E2a promoter independent of E1a and E1b. The consequences of E2a expression in human airway epithelial cells are unknown. Nonhuman primates administered the *lacZ* and CFTR adenoviruses into the airway express high levels of DBP in a subset of *lacZ* expressing cells but do not generate an immune response to this protein product of the E2a gene (data not shown).

Xenografts were sequentially irrigated for a 3 week period after

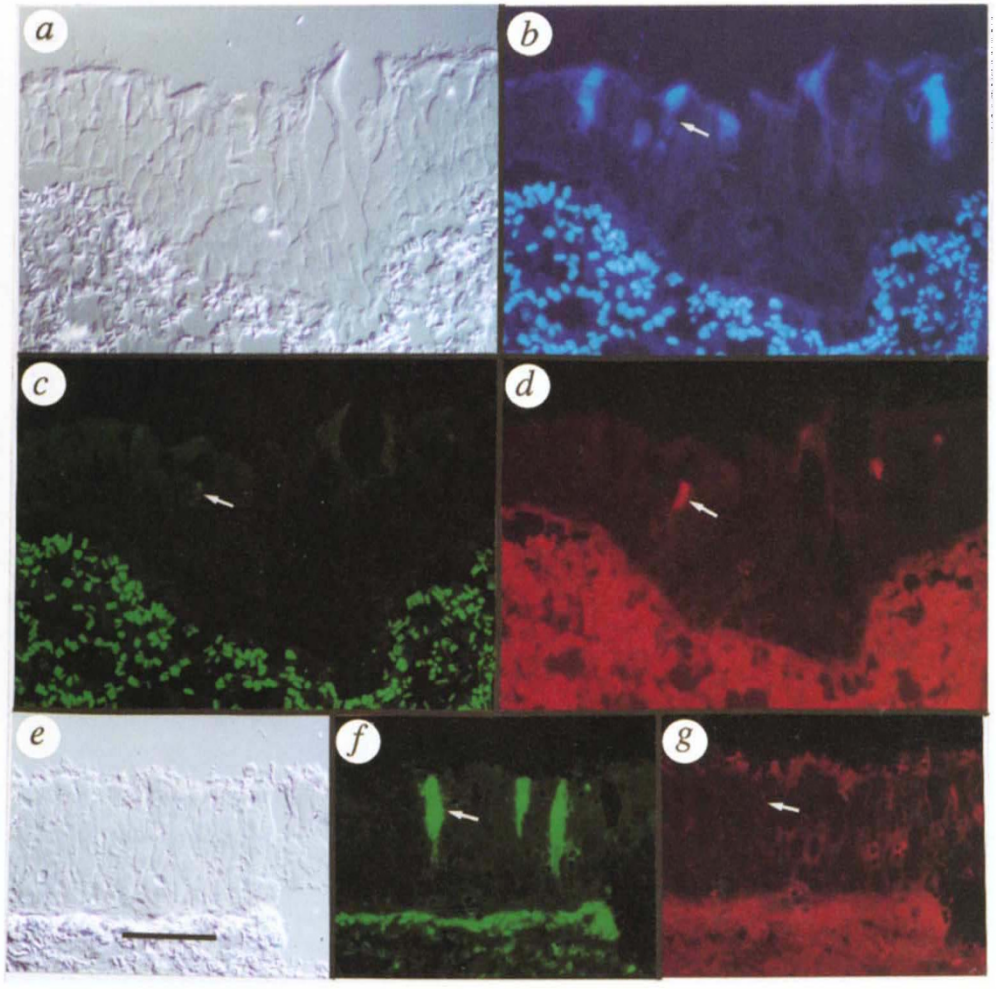


Fig. 6 Immunocytochemical detection of adenoviral proteins. Frozen sections (6 μm) of xenografts infected with 10^{11} pfu ml^{-1} Ad.CMV*lacZ* and harvested 3 days post infection were analysed by triple immunofluorescence (Nomarski, *a*) with antibodies to β-galactosidase detected with AMCA (*b*), fibre detected with texas red (*c*) and DBP detected with FITC (*d*). *e–g* show a xenograft infected with wild type Ad5, harvested 20 h postinfection and analysed by double immunofluorescence (Nomarski, *e*) with antibodies to fibre (*f*) and DBP (*g*). Mock infected xenografts analysed by triple immunofluorescence with the above antibodies showed no immunoreactive staining (data not shown). Arrows in *b–d* indicate a cell expressing both β-galactosidase and DBP but not fibre. Arrows in *f* and *g* indicate a cell expressing both fibre and DBP. Scalebar, 50 μm.

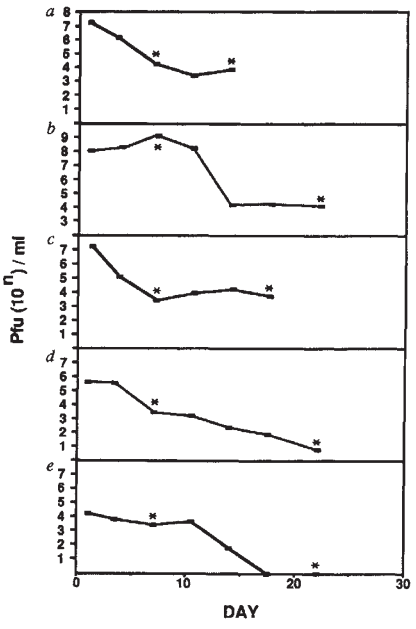


Fig. 7 Recovery of recombinant virus in xenograft effluents. Effluents (1 ml) were collected at 3 1/2 day intervals from xenografts infected with Ad.CMV*lacZ* and were titered by Xgal stained pfu assay on 293 cells. All plaques generated on 293 cells contained β-galactosidase as evident by blue precipitate. Recovered virus is plotted on a log scale versus the time after infusion of virus measured in days. Following the completion of the experiment, the xenografts were harvested, xgal stained and evaluated for % genetic reconstitution in the surface epithelial cells: *a–c*, 5–20% *lacZ* positive cells; *d*, 1% *lacZ* positive cells; and *e*, less than 0.01% *lacZ* positive cells. Asterisks mark effluents that were assayed for wild type adenovirus by the ability to cause cytopathic effects on Hela cells.

exposure to adenovirus, and the effluents were analysed for wild type and recombinant virus. Wild type virus was never detected in the effluents and the concentration of recombinant virus dropped precipitously during the initial week and stabilized at low but detectable concentrations for the second phase of the experiment which lasted up to 24 days. It is possible that the virus recovered in the effluents represents residual virus from the initial infusion. An alternative explanation is that the genetically reconstituted xenografts are supporting low levels of virus production. Replication of E1a-deleted viruses has been described *in vitro*[27]. One potential mechanism to account for the presence of virus in effluents is that an occasional cell in the xenograft overcomes the block in adenoviral replication leading to its death and the production of more recombinant virus. The life cycle of group C viruses such as Ad2 and Ad5 in the context of full E1 expression is extremely efficient and results in the production of 10,000 virion per infected cells[29]. Progression of the full Ad lytic cycle in only 1–2 cells per xenograft per week could account for the steady state level of recombinant virus detected in the effluents (100 to 10,000 viruses/irrigation). Another potential mechanism is that the virus replicates at low levels in a large population of infected cells and low quantities of virus are released when the cells undergo normal turnover. The inability to detect wild type levels of structural proteins in a sample of 1,500 infected cells is consistent with either mechanism.

An important outcome of these studies as it relates to the utility of recombinant adenoviruses for gene therapy is the stability of recombinant gene expression that was achieved in the human xenograft. Detailed characterization of the molecular state of the viral genome in xenografts is difficult because of the limited amount of material available for analysis. DNA analysis of cultured human epithelial cells infected with the *lacZ* and CFTR viruses indicated that the adenoviral genome persists primarily as nonintegrated DNA (data not shown). Also, episomal persistence of recombinant Ad.RSVβgal has been shown in newborn mice[30]. This suggests that the persistence achieved in the xenograft may be due to extrachromosomal viral genomes that are stabilized or replicating in the absence of virus formation. An alternative explanation is that the apparent persistence of recombinant gene expression is due to ongoing production of virus and reinfection. This is unlikely because the levels of virus recovered in the effluents is 5 to 6 logs lower than what is necessary to sustain the observed level of stable genetic reconstitution.

In summary, we describe a xenograft animal model for studying the feasibility and biology of viral-mediated gene transfer in an intact human airway epithelium. Based, in part, on the findings in human xenografts, we submitted and received approval from the Recombinant DNA Advisory Committee to begin a phase I trial of Ad.CB-CFTR in patients. The ultimate utility of E1-deleted adenoviruses in the treatment of CF lung disease must await additional preclinical and clinical studies.

Methodology

Preparation of recombinant adenovirus. Four different replication defective adenoviruses based on Ad5 were used in this study including Ad.E1Δ, Ad.CMV*lacZ*, Ad.CB*CFTR*, and Ad.RSVβgal. Ad.E1Δ has been deleted of E1a sequences spanning 1.0 to 9.2 map units (mu) and E3 sequences spanning 78.4 to 86 mu. A minigene containing the CMV promoter, cytoplasmic *lacZ* gene, and SV40 poly A was introduced at the site of the E1 deletion of Ad.E1Δ to make Ad.CMV*lacZ*. The structure of Ad.RSVβgal has been described previously[30]. In this virus, E1 sequences from 1.3 to 9.4 mu have been deleted and replaced with a minigene containing the Rous sarcoma virus long terminal repeat (RSV LTR), *lacZ* gene with nuclear localization sequences, and SV40 early region polyadenylation signal; in addition E3 sequences spanning to 78.5–84.7 mu have been deleted. Ad.CB*CFTR* is a derivative of Ad.E1Δ in which the following minigene has been inserted into the E1a deletion site: CMV enhancer, β-actin promoter, human CFTR cDNA and SV40 poly A . Detailed descriptions of the viruses Ad.E1Δ, Ad.CMV*lacZ*, and Ad.CB*CFTR* will be provided elsewhere.

Stocks of recombinant viruses were prepared as follows. Cultures of 293 cells (30×150mm plates) grown in DMEM containing 10% fetal calf serum (FCS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin were infected at 80% confluency with an MOI equal to 5 pfu per cell. Cells were harvested 30 h following infection by centrifugation. The pellets were resuspended in a final volume of 18 ml of 10 mM Tris pH 8.0 and subjected to three rounds of freeze–thaw, followed by separation of cell debris by centrifugation at 1,500×g for 20 min. Crude viral supernatants were layered onto a CsCl step gradient and centrifuged for 2 h at 50,000× g. The intact viral particles were subjected to a second round of CsCl banding such that the final CsCl purified adenovirus contained 3–6 x 10^{13} viral particles (as measured by OD at 260 nm) in 500–700 μl. Concentrated viral stocks were desalted by gel filtration through Sephadex G50 in Hams F12 medium to yield a final purified stock of 1–2×10^{13} viral particles ml^{-1}. Viral titres yielded stocks ranging from 0.2–2×10^{12} pfu ml^{-1}. Viral stocks were used for infusion into xenografts immediately after completion of the purification. All stocks were evaluated for the presence of replication competent adenovirus by infection at an MOI of 10 onto HeLa cells and passaging the cells for 30 days. Presence of replication competent virus in the original stock would manifest as the development of cytopathic effects in the HeLa cells. None of the stocks used in these experiments yielded such effects.

Generation of human bronchial xenografts. Primary human bronchial epithelial cells were harvested from the mainstem bronchi of lungs (at least three samples for each vector analysed) destined for transplantation essentially as described[31]. Dissected airways were rinsed with MEM containing 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 40 μg ml^{-1} tobramycin, 50 μg ml^{-1} ceftazidine, 2.5 μg ml^{-1} amphotericin B, 10 μg ml^{-1} DNAse, and 0.5 mg ml^{-1} DTT for 4–12 h at 4 °C. Tissue was then placed in the same media supplemented with 0.1% protease-14 and incubated for an additional 30–34 h at 4 °C. Following the addition of FCS to a final concentration of 10%, the cells were harvested by agitation and blunt scraping. Cells were

pelleted and washed twice in Hams F12 containing 10% FCS and plated at 2×10^6 cells/100 mm dish in Hams F12 containing 1 μM hydrocortisone, 10 μg ml^{-1} insulin, 30 nM thyroxine, 5 μg ml^{-1} transferrin, 25 ng ml^{-1} epidermal growth factor, 3.75 μg ml^{-1} endothelial cell growth supplement, 10 ng ml^{-1} cholera toxin, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 40 μg ml^{-1} tobramycin, 50 μg ml^{-1} ceftazidine, and 2.5 μg ml^{-1} amphotericin B. The medium was replaced after 36 h and changed every 24 h thereafter. On the fourth day, cells were harvested by treatment with 0.1% trypsin followed by the addition of 10% FCS/Ham's F12 and resuspended at a concentration of 1×10^6 cells per 25 μl in hormonally defined medium in preparation for seeding.

Open-ended grafts were generated from rat tracheas removed from 200–250 g male Fisher 344 rats and denuded by three rounds of freeze-thaw as described previously[32]. Cells (1×10^6) were injected into the lumen of denuded rat tracheas followed by ligation of the tracheal ends to flexible plastic tubing. These seeded xenografts were transplanted subcutaneously into the flanks of *nu/nu* mice such that the ends of the tubing exited through the back of the neck. Grafts were allowed to regenerate 3–4 weeks before infusion of adenovirus. Stocks of adenoviruses in Hams F12 (1ml) were infused into the xenografts over the course of 1 h and excess fluid was subsequently removed from the lumen by expulsion with air.

Electron microscopic and morphometric analysis of xenografts. Xenografts were excised and fixed as described[32]. Following fixation, the tissue was washed repeatedly in 0.1 M cacodylate, postfixed in 1% osmium tetroxide, dehydrated in alcohols, and embedded in epoxy resins. Sections were stained with uranyl acetate and lead citrate before being viewed and photographed in a Philips CM10 electron microscope. Morphometric analysis of cell types contained within a donor bronchus and three xenografts generated from this tissue were performed to assess the extent of epithelial reconstitution within this xenograft model. Cells were categorized based on morphologic criteria into 4 groups: ciliated cells by possessing apically localized cilia, goblet cells by the presence of electron lucent secretory granules, intermediate cells by no luminal contact with cytoplasm extending at least one third the height of the epithelium and not fulfilling the criteria of basal cells, and basal cells by the presence of tonofilaments and a high nuclear to cytoplasmic ratio with the majority of cytoplasm residing on the basal lamina. At least 30 independent fields from 5 blocks were analysed from donor bronchus to give a total of 1,500 cells. Each of three independently generated xenografts was embedded into four blocks and one complete cross section of each of these blocks was analysed giving a total of 12 independent regions of the xenografts. In total, 3,000 cells were analysed from three xenografts.

Cytochemical and immunocytochemical analysis of xenografts. Cytochemical localization and characterization of grafts for β-galactosidase activity by light microscopy was performed with glutaraldehyde-fixed tissue stained in Xgal for 4 h followed by embedding in GMA as described[32]. The abundance of *lacZ* transgene-expressing cells was quantitated by counting the percentage of xgal positive cells from GMA sections within a group of 16,000 cells. The distribution of the various cell types (ciliated, basal, goblet and intermediate cells) within the xenograft epithelium was based on averages from 3,000 cells counted from 5 independent regions of a representative graft. Identification of the various cell types was based on the following morphologic criteria: ciliated cell, the presence of cilia; basal cell, cuboidal appearing cell of high nuclear to cytoplasmic ratio with nuclei in the lowest layer of epithelium, direct contact with the basal lamina, and no luminal contact; goblet cells, the presence of mucous granules as visualized under Nomarski optics; and intermediate cells, cells in contact with the basal lamina but with cytoplasm extending upward into the epithelium but not contacting the luminal surface. The relative infectivity of ciliated cells, basal cells, goblet cells, and intermediate cells was quantitated by counting 1,000 Xgal positive cells from GMA sections of grafts infected with 10^{12} pfu ml^{-1} of virus. Distribution of cells expressing *lacZ* was also evaluated by immunocytochemical colocalization with a cell specific markers to basal cells, cytokeratin 14, and one to differentiated columnar cells, cytokeratin 18 (refs 33,34).

Immunocytochemical colocalization of β-galactosidase, cytokeratin 14, and cytokeratin 18 proteins was performed as follows. Sections of fresh frozen tissue (6 μm) were postfixed in methanol for 10 min, air dried, and blocked in PBS containing 20% donkey serum (DS) for 30 min. Sections were then incubated sequentially in undiluted hybridoma supernatant to cytokeratin 14 (gift from F.C.S. Ramaekers, RCK107) for 90 min followed by three 8 min washes in 1.5% DS/PBS and incubation in 5 μg ml^{-1} of AMCA-antimouse Fab2 secondary antibody for 30 min. After washing, these sections were incubated in PBS/1.5% DS containing 66 μg ml^{-1} rabbit anti-β-galactosidase (5'-3' inc.) and FITC-cytokeratin 18 (Sigma) at a dilution of 1:400 for 90 min. Sections were washed and incubated in 5 μg ml^{-1} donkey anti-rabbit texas red for 30 min. Following three washes in 1.5% DS/PBS, sections were mounted in Citifluor antifadent and visualized on a Microphet-FXA Nikon fluorescent microscope. Cell types expressing *lacZ* were quantitated from sections stained for β-galactosidase, cytokeratin 14, and cytokeratin 18; 1,000 total *lacZ* positive cells were counted. Localization of CFTR was performed using a previously describes antibody to the 13 C-terminal amino acids of human CFTR (α1468) as described[15].

Immunocytochemical colocalization of β-galactosidase with the adenoviral proteins DBP, fibre, and hexon was performed as with cytokeratin colocalization using the following modifications. Sections were incubated sequentially with 66 μg ml^{-1} rabbit anti-β-galactosidase (5' to 3' Inc), a 1/10 dilution of hybridoma supernatant to Ad5 DBP[35], 5 μg ml^{-1} of both donkey anti-rabbit-AMCA and donkey anti-mouse-texas red, followed by a 1/10 dilution of mouse anti-Ad3 fibre-FITC (Ab805F, Chemicon). Western analysis of purified adenovirus type 5 indicated that Ab805F recognizes a 62 kd protein consistent with fibre protein (data not shown). Additional sections were treated similarly by replacing the Ab805F with a goat anti-Ad5-FITC antibody to the hexon protein of adenovirus type 5 (Ab1059F, Chemicon).

In situ detection of CFTR mRNA. Frozen sections (6 μm) were mounted on gelatin poly(L-lysine)-coated slides and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for *in situ* analysis as previously described using S^{35} RNA probes to the R-domain (1,899 to 2,623 bp) of human CFTR[15]. Sense and RNAse pretreatment with antisense probes were used as controls for hybridization specificity.

Recovery of adenoviruses from xenografts. To assess the ability of recombinant adenovirus to replicate within human xenograft epithelium, effluent fractions were collected at timed intervals following infection. Xenografts were infected with freshly prepared stocks of virus (10^{11}, 10^{10}, and 10^9 pfu ml^{-1}) for 16 h followed by washing with two 1ml aliquots of buffered saline. The second aliquot was designated fraction 1. At 3 1/2 day intervals additional fractions were collected by irrigating the lumen of the xenograft with 1 ml aliquots of buffered saline. All fractions were frozen on dry ice and stored at -80 °C. Upon completion of the experiment the fractions were thawed and evaluated for recombinant virus by a limiting dilution plaque assay on 293 cells. Plaques were were stained for β-galactosidase by overlaying 1ml of Xgal solution onto the agar at day 9 following infection. All plaques showed the presence of blue Xgal precipitate.

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