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Adenovirus-Mediated Gene Transfer Transiently Corrects the Chloride Transport Defect in Nasal Epithelia of Patients with Cystic Fibrosis

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Summary

To evaluate the potential of direct transfer of cystic fibrosis transmembrane conductance regulator (CFTR) cDNA for the treatment of cystic fibrosis (CF), we administered an E1-deficient adenovirus, encoding CFTR, to a defined area of nasal airway epithelium of three individuals with CF. This treatment corrected the Cl^- transport defect that is characteristic of CF-affected epithelia. After treatment, there was a decrease in the elevated basal transepithelial voltage, and the normal response to a cAMP agonist was restored. We found no evidence of viral replication or virus-associated adverse effects, even at the highest dose tested (25 MOI). These data represent a small step in achieving long-term improvement of CF lung function by gene therapy.

Introduction

Cystic fibrosis (CF) is a common, autosomal recessive disease (Boat et al., 1989) caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) (Riordan et al., 1989). Recent work has shown that CFTR is a Cl^- channel that is regulated by cAMP-dependent phosphorylation and by intracellular nucleotides (Welsh et al., 1992; Collins, 1992; Riordan, 1993). Mutations in the CFTR gene (Tsui, 1992) have been shown to cause a loss of function of CFTR Cl^- channels (Welsh and Smith, 1993), thereby producing the hallmark of the disease: defective cAMP-mediated Cl^- transport across affected epithelia (Quinton, 1990; Boat et al., 1989). Although this leads to a variety of clinical manifestations, it is lung disease, and specifically disease of the pulmonary airways, that is the major cause of CF-associated morbidity. Despite progress, no current therapy treats the basic defect, and consequently, CF remains a life-threatening and often lethal disease. However, recently obtained knowledge of the gene that encodes CFTR, an understanding of the function and biochemistry of the protein product, and insight into the molecular basis of the disease (Welsh et al., 1992; Collins, 1992; Riordan, 1993; Welsh and Smith, 1993) all now suggest that gene transfer could represent an important advance in treatment.

The feasibility of gene transfer to lung cells was initially demonstrated by our finding that expression of the cDNA for wild-type CFTR, but not mutant CFTR, corrected the Cl^- channel defect in primary cultures of CF airway epithelia (Rich et al., 1990). Similar results were obtained with pancreatic epithelial cells (Drumm et al., 1990). Complementation of the CF defect has since been reported using a variety of different vectors and delivery systems. Most recently, delivery of CFTR cDNA complexed with cationic lipids to epithelia of mice bearing a disrupted CFTR gene was reported to correct the Cl^- transport defect (Hyde et al., 1993).

The target tissue for gene transfer that is most likely to be of therapeutic benefit in CF patients is the airway epithelium. Because removal of airway epithelial cells, transfer of cDNA in vitro, and reimplantation of the cells into the lungs appear impractical, gene therapy will require treatment of airway cells in vivo. Adenovirus-based vectors (Graham and Prevec, 1992; Berkner, 1988) appear promising for gene transfer to CF airway epithelia since they have been used to express CFTR in airway epithelia in vitro and in animals (Chasse et al., 1989; Rosenfeld et al., 1992; Engelhardt et al., 1993; Rich et al., 1993; Mastrangeli et al., 1993; Zabner et al., 1993). These studies are encouraging not only in terms of the ability to express recombinant protein, but also in terms of the safety profile at the doses tested.

However, assessment of the value of adenovirus as a delivery vector for gene therapy of CF requires studies in humans. Considering the limitations of in vitro and animal studies and the uncertainties of using adenovirus vectors in humans, we adopted a conservative strategy in designing an experimental protocol. We elected to examine the efficacy and safety of an adenovirus vector in the nasal epithelia of patients with CF because this tissue has a morphology and function similar to those of intrapulmonary airways and because nasal epithelium manifests the CF Cl^- transport defect (Knowles et al., 1981, 1983; Widdicombe et al., 1985; Welsh, 1987). The use of nasal epithelium confers several advantages for initial studies. First, the epithelium is accessible for vector administration, inspection, and sampling, thereby allowing noninvasive evaluation. Second, the risk to participants is minimized if an adverse reaction should occur. Third, the effect of expressing CFTR Cl^- channels in the apical membrane of the epithelium is readily assessed by measuring the in vivo transepithelial voltage (Knowles et al., 1981; Alton et al., 1987) and its response to cAMP agonists. In this way the protocol allowed us to evaluate directly the ability of a recombinant adenoviral vector to correct defective transepithelial Cl^- transport in humans.

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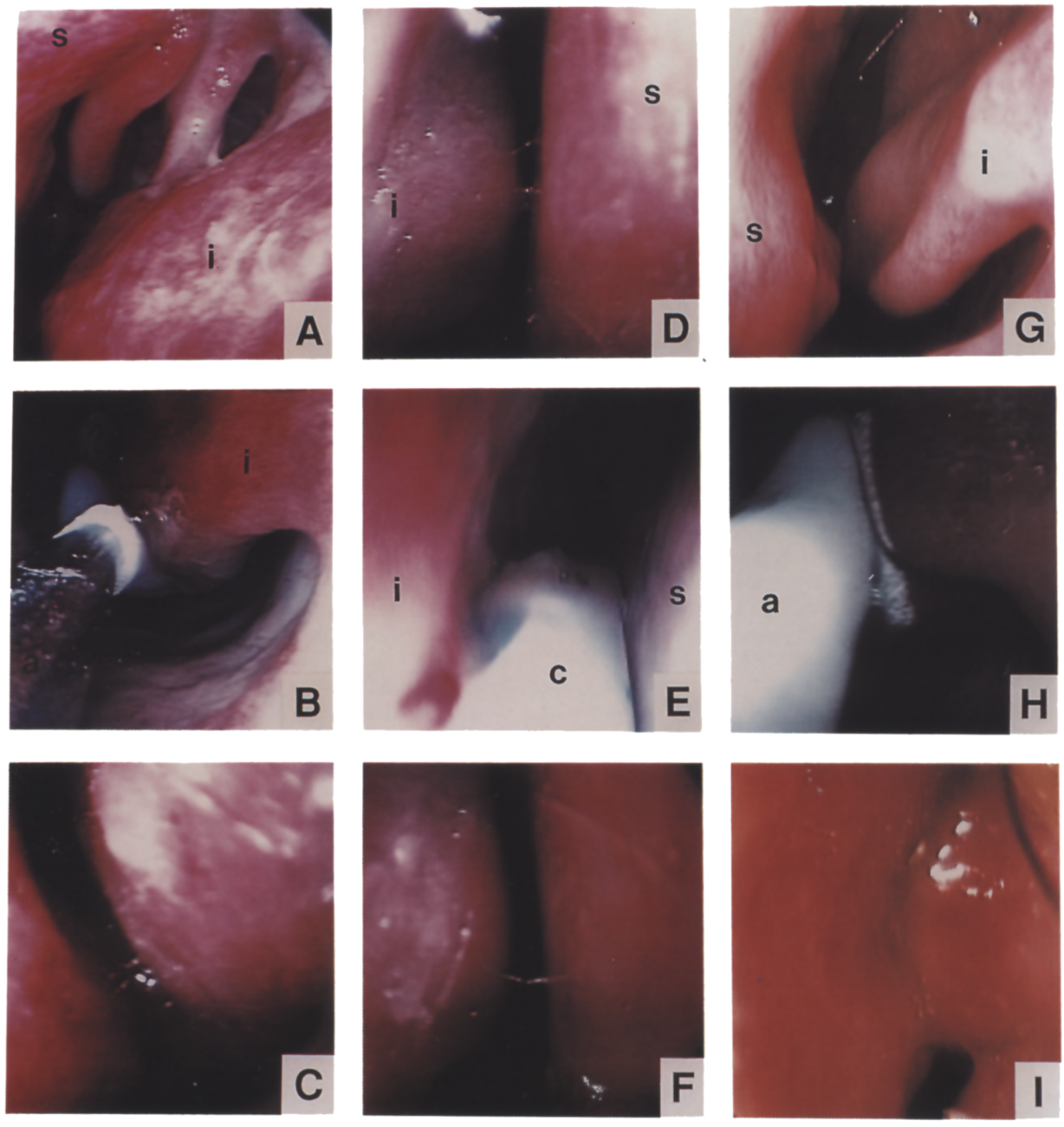

Figure 1. Photographs of Nasal Mucosa

Photographs of nasal mucosa immediately before (A, D, and G), during (B, E, and H), and 1 day after (C, F, and I) the application procedure. First column is from the right nostril of the third patient; note the applicator placed against the medial surface of the inferior turbinate in (B). Second column is from left nostril of third patient; note the rubber catheter touching medial surface of the inferior turbinate in (E). Third column is from a CF patient treated with the identical procedures except that saline was administered instead of Ad2/CFTR-1; note the applicator in (H). (C) and (I) show similar erythema, edema, and exudate, whereas none is observed in (F). The lowercase letters refer to the following anatomical structures: s, septum; i, inferior turbinate; a, applicator; and c, Foley catheter.

Results

We limited application of recombinant adenovirus to a defined area of nasal epithelium by using a small plastic applicator (Figure 1B). After local anesthesia and vasoconstriction of the nasal mucosa, a solution containing adenovirus type 2 (Ad2)/CFTR-1 was applied to approximately 0.5 cm^2 of the medial surface of both inferior turbinates, maintained in place for 30 min, and then removed by suction. One or three days later, the nasal mucosa treated with Ad2/CFTR-1 was removed by biopsy on one side and the mucosa on the opposite side was evaluated with electrical measurements, swabs, and brushings. The application procedure had the advantage of minimizing the total

dose of Ad2/CFTR-1, and equally importantly, it allowed us to define the approximate multiplicity of infection (MOI, the number of infectious units [IU] per cell). We studied three patients: the first received 2×10^6 IU, the second received 6×10^6 IU, and the third received 5×10^7 IU. Assuming that there are 2×10^6 cells per cm^2 in the nasal epithelium (Mariassy, 1992) the approximate MOIs were 1, 3, and 25, respectively.

One day after Ad2/CFTR-1 administration and at all subsequent time points, we were unable to culture Ad2/CFTR-1 from the nasal epithelium, pharynx, blood, urine, or stool. As a control for the sensitivity of the culture assay, we routinely spiked samples with 10 and 100 IU of Ad2/CFTR-1. In every case, the spiked samples were positive, indicating that, at a minimum, we should have been able to detect 10 IU of Ad2/CFTR-1. We observed no evidence of a systemic response as assessed by history, physical examination, serum chemistries or cell counts, chest and sinus X-rays, pulmonary function tests, or arterial blood gases performed before and after Ad2/CFTR-1 administration. We were not able to detect an increase in antibodies to adenovirus by enzyme-linked immunosorbent assay or by neutralization for 35 days after treatment, although we have detected an increase in antibodies in similarly treated monkeys and cotton rats (Zabner et al., 1993). In the animal experiments, however, higher doses were used and the animals were seronegative before administration.

Three to four hours after Ad2/CFTR-1 administration, at the time that local anesthesia and localized vasoconstriction abated, all patients began to complain of nasal congestion and in one case, mild rhinorrhea. These were isolated symptoms that had diminished by 18 hr and were resolved by 28 to 42 hr. Inspection of the nasal mucosa showed mild to moderate erythema, edema, and exudate (Figures 1A–1C). These physical findings followed a time course similar to the symptoms. The physical findings were not limited to the site of virus application, even though preliminary studies using the applicator showed that marker methylene blue was limited to the area of application. In two additional patients with CF, we used the identical anesthesia and application procedure, but applied saline instead of virus, yet the same symptoms and physical findings were observed in these patients (Figures 1G–1I). Moreover, the local anesthesia and vasoconstriction generated similar changes even when we did not use the applicator (data not shown), suggesting that the anesthesia/vasoconstriction caused some, if not all, of the injury. Twenty-four hours after the application procedure, analysis of cells removed by nasal swabs revealed an equivalent increase in the percentage of neutrophils in patients treated with Ad2/CFTR-1 or with saline. One week after application, the neutrophilia had resolved in both groups. Respiratory epithelial cells obtained by nasal brushing appeared normal at 1 week and at subsequent time points and showed no evidence of inclusion bodies. To evaluate the mucosa further, the epithelium was biopsied on day 3 in the first patient and day 1 in the second patient. Independent evaluation by two pathologists not otherwise associated with the study suggested changes consistent with mild trauma and possible ischemia (probably secondary to the anesthetic/vasoconstrictors used before virus administration), but there were no abnormalities suggestive of virus-mediated damage.

Because the application procedure produced some mild injury in the first two patients, we altered the method of administration in the third patient. We used a method that would not need local anesthesia or vasoconstriction and that was thus less likely to cause injury, but that was also less certain in its ability to constrain Ad2/CFTR-1 to a precisely defined area. On the right side, we administered Ad2/CFTR-1 as we had in the first two patients, and on the left side we administered the virus without anesthesia or the applicator, instead using a small Foley catheter to apply and maintain Ad2/CFTR-1 in a relatively defined area by surface tension (Figure 1E). On the right side, the symptoms and physical findings were the same as those observed in the first two patients. By contrast, on the left side there were no symptoms, and on inspection the nasal mucosa appeared normal (Figures 1D–1F). Nasal swabs obtained from the right side showed neutrophilia similar to those observed in the first two patients. In contrast, the left side, which had no anesthesia and had minimal manipulation, did not develop neutrophilia. Biopsy of the left side on day 3 after administration (Figure 2) showed morphology consistent with CF—a thickened basement membrane and occasional polymorphonuclear cells in the submucosa—but no abnormalities that could be attributed to the adenovirus vector.

The first patient developed symptoms of a sore throat and increased cough that began 3 weeks after treatment and persisted for 2 days. Six weeks after treatment she developed an exacerbation of her bronchitis/bronchiectasis and hemoptysis that required hospitalization. The second patient had a transient episode of minimal hemoptysis 3 weeks after treatment; it was not accompanied by any other symptoms before or after the episode. The third patient had an exacerbation of bronchitis 3 weeks after treatment, for which she was given oral antibiotics. Based on each patient's pretreatment clinical history, evaluation of the episodes, and viral cultures, we could discern no evidence that linked these episodes to administration of Ad2/CFTR-1. Rather, the episodes appeared consistent with the normal course of disease in each individual.

The loss of CFTR Cl^- channel function causes abnormal ion transport across affected epithelia, which in turn contributes to the pathogenesis of CF-associated airway disease (Boat et al., 1989; Quinton, 1990). In airway epithelia, ion transport is dominated by two electrically conductive processes: amiloride-sensitive absorption of Na^+ from the mucosal to the submucosal surface and cAMP-stimulated Cl^- secretion in the opposite direction (Quinton, 1990; Welsh, 1987). These two transport processes can be assessed noninvasively by measuring the voltage across the nasal epithelium (Vt) in vivo (Knowles et al., 1981; Alton et al., 1987). Figure 3 shows an example from a normal subject. Under basal conditions, Vt was electrically negative (lumen referenced to the submucosal surface). Perfusion of amiloride (100 μM) onto the mucosal surface inhibited Vt by blocking apical Na^+ channels (Knowles et al., 1981; Quinton, 1990; Welsh, 1987). Subsequent perfusion

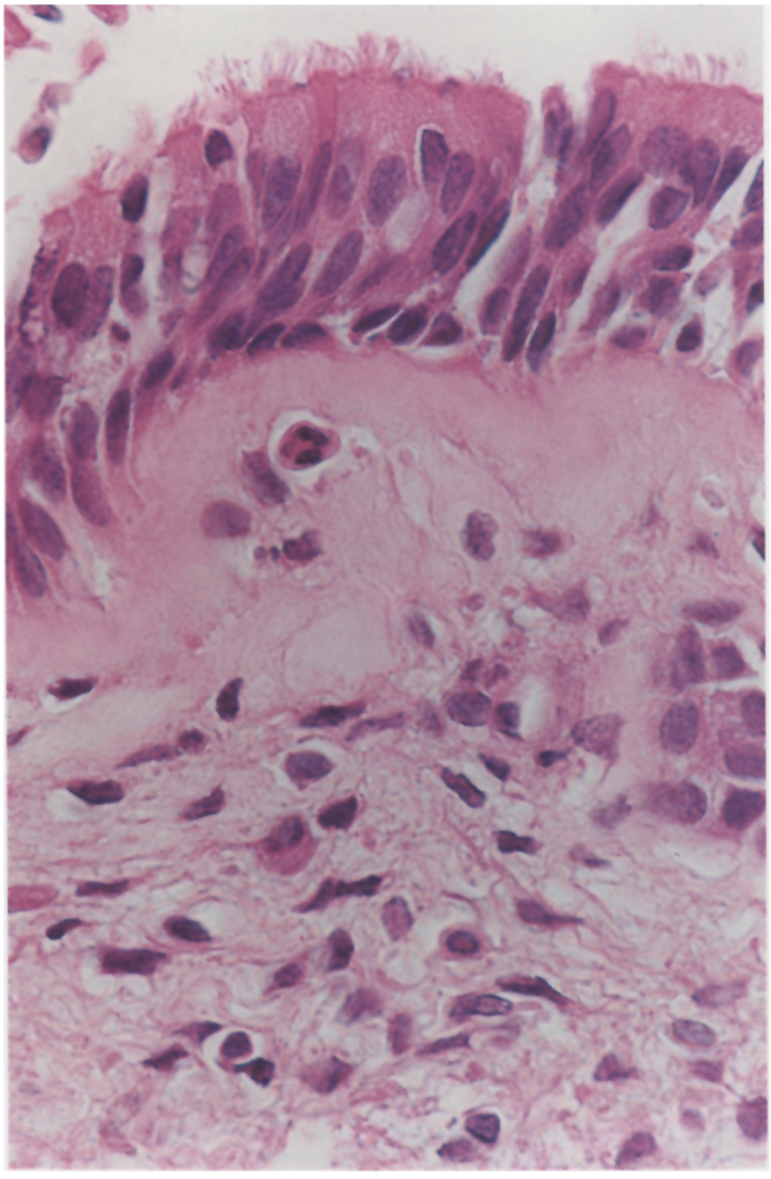

Figure 2. Photomicrograph of Hematoxylin- and Eosin-Stained Biopsy of Nasal Mucosa Obtained from the Third Patient 3 Days after Ad2/CFTR-1 Administration

Width of figure is 160 μm.

with terbutaline (10 μM), a β-adrenergic agonist, hyperpolarized Vt by increasing cellular levels of cAMP, opening CFTR Cl^- channels, and stimulating Cl^- secretion (Quinton, 1990; Welsh et al., 1992). Figure 4A shows results from seven normal subjects: basal Vt was −10.5 ± 1.0 mV, and in the presence of amiloride, terbutaline hyperpolarized Vt by −2.3 ± 0.5 mV.

In patients with CF, Vt was more electrically negative than in normal subjects (Figure 4B), as has been previously reported (Knowles et al., 1981). Basal Vt was −37.0 ± 2.4 mV, much more negative than values in normal subjects ($p < 0.001$). (Note the difference in scale in Figures 4A and 4B.) Amiloride inhibited Vt, as it did in normal subjects. However, when we perfused terbutaline onto the epithelium in the presence of amiloride, Vt failed to hyperpolarize. Instead, Vt either did not change or became less negative: on average Vt depolarized by +1.8 ± 0.6 mV, a result very different from that observed in normal subjects ($p < 0.001$).

After we applied Ad2/CFTR-1, basal Vt became less negative in all three CF patients; Figure 5 shows an example from the third patient before (Figure 5A) and after (Figure 5B) treatment and Figures 6A, 6C, and 6E show the time course of changes in basal Vt for all three patients. The decrease in basal Vt suggests that application of Ad2/CFTR-1 corrected the CF electrolyte transport defect in nasal epithelium of all three patients. Additional evidence came from an examination of the response to terbutaline. Figure 5B shows that in contrast with the response before we applied Ad2/CFTR-1, after virus application, in the presence of amiloride, terbutaline stimulated Vt. Figures 6B, 6D, and 6F show the time course of the response. These data indicate that Ad2/CFTR-1 corrected the CF defect in Cl^- transport. Correction of the Cl^- transport de-

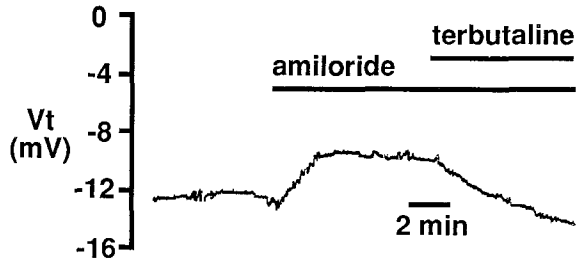

Figure 3. Measurement of Vt across the Nasal Epithelium of a Normal Subject

Amiloride (100 μM) and terbutaline (10 μM) were perfused onto the mucosal surface during times indicated by the bars.

fect cannot be attributed to the anesthesia/application procedure because it did not occur in patients treated with saline instead of Ad2/CFTR-1 (Figure 7). Moreover, the effects of the anesthesia were generalized on the nasal mucosa, but basal Vt decreased only in the area of virus administration. Finally, we observed similar changes in the left nasal mucosa of the third patient (Figures 6E and 6F), which had no symptomatic or physical response after the modified application procedure.

We attempted to detect CFTR transcripts by reverse transcriptase-polymerase chain reaction (PCR) and by immunocytochemistry in cells from nasal brushings and biopsies. These attempts were not successful. Although we had been successful with similar studies in animals (Zabner et al., 1993), those studies used much higher doses of Ad2/CFTR-1. Our lack of success likely reflects the small amount of available tissue, the low MOI, the fact that only a fraction of cells may have been corrected, and the fact that Ad2/CFTR-1 contains a low to moderate strength promoter (E1a) that produces much less mRNA and protein than comparable constructs using a much stronger cytomegalovirus promoter (unpublished data). We had chosen the E1a promoter because CFTR is normally expressed at very low levels in airway epithelial cells (Trapnell et al., 1991). It is also difficult to detect CFTR protein and mRNA in normal human airway epithelia, although function is readily detected because a single ion channel can conduct a very large number of ions per second and thus efficiently support Cl^- transport.

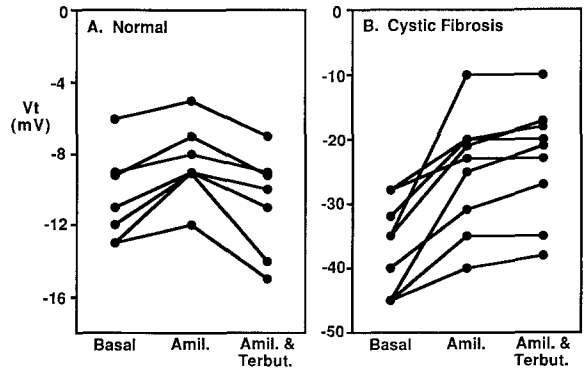

Figure 4. Vt across the Nasal Epithelium of Normal Subjects and Patients with CF

(A) Normal subjects. (B) Patients with CF. Note the difference in scale in (A) and (B). Values were obtained under basal conditions, during perfusion with amiloride (100 μM) and during perfusion of amiloride plus terbutaline (10 μM) onto the mucosal surface. Data are from seven normal subjects and nine patients with CF.

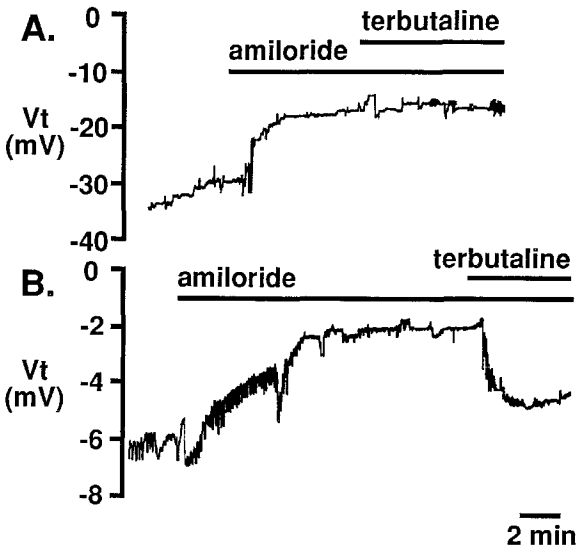

Figure 5. Measurement of Vt across the Nasal Epithelium of the Third Patient before and 7 Days after Administration of ~25 MOI of Ad2/CFTR-1

Before (A) and 7 days after (B) administration of Ad2/CFTR-1. Note the difference in scale in (A) and (B). Amiloride and terbutaline were perfused onto the mucosal surface during times indicated.

With time, the electrical changes that indicate correction of the CF defect reverted toward pretreatment values. However, the basal Vt appeared to revert more slowly than did the change in Vt produced by terbutaline. We do not know the significance of this difference, but it may reflect the relative sensitivity of the two measurements to expression of normal CFTR. In any case, this study was not designed to test the duration of correction because the treated area was removed by biopsy on one side and the nasal mucosa on the other side was brushed to obtain cells for analysis at 7 to 10 days after virus administration and then at approximately weekly intervals. Brushing the mucosa removes cells, disrupts the epithelium, and reduces basal Vt to 0 for at least 2 days afterward, thus preventing an accurate assessment of duration of the effect of Ad2/CFTR-1.

Discussion

Efficacy of Adenovirus-Mediated Gene Transfer

The major conclusion of this study is that in vivo application of a recombinant adenovirus encoding CFTR can correct the defect in airway epithelial Cl^- transport that is characteristic of CF epithelia.

Complementation of the Cl^- channel defect in human nasal epithelium could be measured as a change in basal voltage and as a change in the response to cAMP agonists.

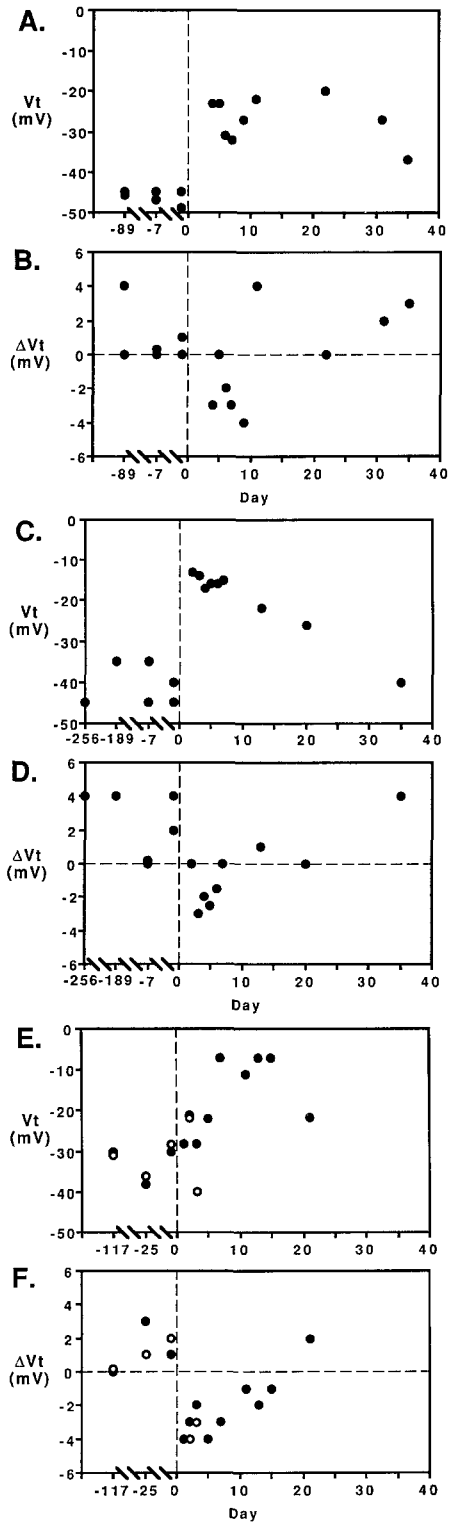

Figure 6. Time Course of Changes in Transepithelial Electrical Properties before and after Administration of Ad2/CFTR-1

(A) and (B) are from the first patient who received approximately 1 MOI; (C) and (D) are from the second patient who received approximately 3 MOI; and (E) and (F) are from the third patient who received approximately 25 MOI. (A), (C), and (E) show values of basal Vt and (B), (D), and (F) show the change in Vt (ΔVt) produced by perfusion of terbutaline in the presence of amiloride. Day 0 indicates the day of AD2/CFTR-1 administration. For the third patient, closed symbols are from the right side and open symbols are from the left side, which was removed by biopsy on day 3. For each patient, we compared values of basal Vt and ΔVt before treatment with values obtained for the first 7 days (the number of days before the epithelium was brushed) after treatment. Electrical properties were significantly different after treatment: for the first patient, Vt, $p < 0.001$ and ΔVt, $p = 0.008$; for the second patient, Vt, $p < 0.001$ and ΔVt, $p = 0.002$; and for the third patient, Vt, $p = 0.02$ and ΔVt, $p < 0.001$.

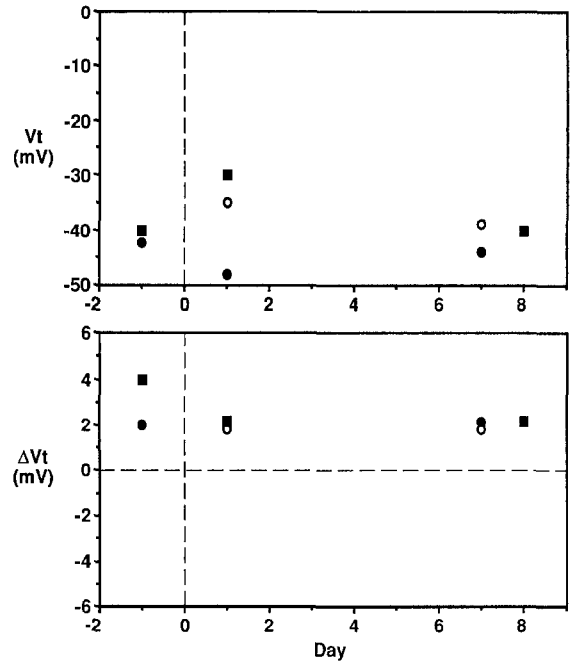

Figure 7. Time Course of Changes in Transepithelial Electrical Properties before and after Administration of Saline Instead of Vector to CF Patients

Day 0 indicates the time of mock administration. (A) shows basal Vt and (B) shows the change in Vt following perfusion of terbutaline in the presence of amiloride (ΔVt). Closed symbols are data from two patients that received local anesthetic/vasoconstriction and placement of the applicator for 30 min. Open symbol is data from a patient that received local anesthetic/vasoconstriction, but not placement of the applicator. Symptomatic changes and physical findings were the same as those observed in CF patients treated with a similar administration procedure and Ad2/CFTR-1. Nasal mucosa of patient indicated by closed squares is that shown in Figures 1G–1I.

Although the protocol was not designed to establish duration, changes in these parameters were detected for at least 3 weeks. These results represent the first report that administration of a recombinant adenovirus to humans can correct a genetic lesion as measured by a functional assay. Our study contrasts with most earlier attempts at gene transfer to humans, in that we administered a recombinant viral vector directly to humans, rather than using an in vitro protocol involving removal of cells from the patient and transduction of the cells in culture, followed by reintroduction of the cells into the patient.

We obtained evidence that the CF Cl^- transport defect was corrected at all three doses of virus, corresponding to 1, 3, and 25 MOI. This result is consistent with our earlier studies showing that similar MOIs reversed the CF fluid and electrolyte transport defects in primary cultures of CF

airway cells grown as epithelia on permeable filter supports (Rich et al., 1993; J. Z. et al., submitted): at an MOI of less than 1, we partially restored cAMP-stimulated Cl^- secretion, and after treatment with 1 MOI Ad2/CFTR-1 cAMP agonists stimulated fluid secretion that was within the range observed in epithelia from normal subjects. At an MOI of 1, a related adenovirus vector produced β-galactosidase activity in 20% of infected epithelial cells as assessed by fluorescence-activated cell analysis (J. Z. et al., submitted). Such data would imply that the pharmacologic dose of adenovirus in CF airways might correspond to an MOI of 1. If we estimate that there are 2×10^6 cells per cm^2 in the airway (Mariassy, 1992), and that the airways from the trachea to the respiratory bronchioles have a surface area of 1400 cm^2 (Weibel, 1963), then there would be approximately 3×10^9 potential target cells. Assuming a particle to IU ratio of 100, this would correspond to approximately 3×10^{11} particles of adenovirus with a mass of approximately 75 μg. While obviously only a crude estimate, such information is useful in designing animal experiments to establish the likely safety profile of a human dose.

It is possible that an efficacious MOI of recombinant adenovirus could be less than the lowest MOI tested here. Some evidence suggests that not all cells in an epithelial monolayer need to express CFTR to correct the CF electrolyte transport defects. Mixing experiments showed that when perhaps 5%–10% of cells overexpress CFTR, the monolayer exhibits wild-type electrical properties (Johnson et al., 1992). Studies using liposomes to express CFTR in mice bearing a disrupted CFTR gene also suggest that only a small proportion of cells need to be corrected (Hyde et al., 1993). Our own results (referred to above) using airway epithelial monolayers and multiplicities of Ad2/CFTR-1 as low as 0.1 showed measurable changes in Cl^- secretion (Rich et al., 1993; J. Z. et al., submitted).

Given the very high sensitivity of electrolyte transport assays (which result because a single Cl^- channel is capable of transporting large numbers of ions per second) and the low activity of the E1a promoter used to transcribe CFTR, our inability to detect CFTR protein and CFTR mRNA is perhaps not surprising. Although we could not detect CFTR mRNA by reverse transcriptase-PCR, we were able to detect Ad2/CFTR-1 DNA in the samples by standard PCR (not shown), demonstrating the presence of input DNA and suggesting that the reverse transcriptase reaction may have been suboptimal. This could have occurred because of factors in the tissue that inhibit the reverse transcriptase. Although there is little doubt that the changes in electrolyte transport measured here result from expression of CFTR, it remains to be seen whether this will lead to measurable clinical changes in lung function. Indeed demonstrating that changes such as those measured here result in meaningful clinical benefit remains one of the major unknowns of this work.

A final reason for our attempts to relate our observations to the MOI involves safety considerations. Even when we have established an effective MOI, it will be impossible to ensure an even distribution of delivered adenovirus to all areas of the airway, especially in a CF patient in which localized regions of airway obstruction are to be expected. Certain treated areas will likely be exposed to amounts of virus higher than the desired, calculated MOI. Safety and efficacy studies over a wide controlled range of MOI are essential if we are to be able to predict the likely consequences of such uneven virus distribution.

Safety Considerations

Application of the adenovirus vector to the nasal epithelium in these three patients was well tolerated. Although we observed mild inflammation in the nasal epithelium of all three patients following administration of Ad2/CFTR-1, similar changes were observed in two volunteers who underwent a sham procedure using saline rather than the viral vector. Clearly a combination of anesthetic- and procedure-related trauma resulted in the changes in the nasal mucosa. We have insufficient evidence to conclude that no inflammation results from virus administration. However, using a modified administration and the highest MOI of virus tested (25 MOI) in one patient, we observed no inflammation under conditions that resulted in evidence of biophysical efficacy that lasted until the area was removed by biopsy at 3 days.

In separate studies we have observed no evidence of inflammation following administration of Ad2/CFTR-1 to a variety of animal species, including nonhuman primates (Zabner et al., 1993). It would not be surprising, however, if an inflammatory response was observed with administration of large doses of an adenovirus vector. For example, in nonpermissive animals (mice) administration of large amounts of adenovirus has been shown to cause lung pathology similar to that observed with wild-type adenoviral infection in cotton rats (Ginsberg et al., 1991). Furthermore, there are unpublished reports of inflammation following administration of high doses of E3-deleted Ad5-based vectors (Dickson, 1993). Clearly these findings indicate that more studies are required to test for the possibility of inflammation in humans. They also highlight the urgent need to determine whether a specific adenovirus construct or some other component of the formulation is responsible for such effects.

In addition, we did not observe evidence of replication of Ad2/CFTR-1. Earlier studies had established that replication of Ad2/CFTR-1 in tissue culture and experimental animals is severely impaired (Rich et al., 1993; Zabner et al., 1993). Replication only occurs in cells that supply the missing early proteins of the E1 region of adenovirus, such as 293 cells, or under conditions where the E1 region is provided by coinfection with or recombination with an E1-containing adenovirus (Graham and Prevec, 1992; Berkner, 1988). The patients studied here were seropositive for adenovirus types 2 and 5 prior to the study, were negative for adenovirus upon culture of nasal swabs prior to administration of Ad2/CFTR-1, and were shown by PCR methods to lack endogenous E1 DNA sequences such as have been reported in some human subjects (Matsuse et al., 1992). Further studies will be required to establish whether all these criteria will need to be met prior to treatment of other patients.

Future Challenges

For the future, many additional questions remain. For example, regarding efficacy, the duration of correction of Cl^- transport is unknown, and additional studies must more precisely address the relationship between MOI, the absolute level of expression, the percentage of cells corrected, and correction of the Cl^- transport defect. The development of an immune response to the vector or to wild-type CFTR might also change efficacy. Questions about safety include the response to increased viral doses and the effect of multiple administrations of vector. The unpublished reports of inflammation noted above are also a concern. All of these issues will need to be addressed before we can conclude that adenovirus-based vectors are likely to be safe and efficacious for treatment of CF. Furthermore, vectors based on adeno-associated virus and receptor-mediated DNA transfer are alternative gene delivery systems. Reports of gene transfer by cationic lipid–DNA complexes are also promising.

Additional studies to answer some of the questions regarding adenovirus and to compare different means of delivery could be performed in the nasal epithelia, but inevitably, other studies will have to be performed using other tissues, including intrapulmonary airway epithelia in CF patients. The major challenge for such studies will be designing protocols that minimize the risk to patients yet at the same time generate evidence of long-term clinical benefit. We emphasize that this study demonstrates transient changes in the electrophysiology of the nasal epithelia and is but one small step toward the goal of long-term improvement in lung function.

Experimental Procedures

Adenovirus Vector

To deliver CFTR cDNA, we used a recombinant adenovirus, Ad2/CFTR-1. The construction and preparation of Ad2/CFTR, and its use in vitro and in vivo in animals, have been previously described (Rich et al., 1993; Zabner et al., 1993). The DNA construct comprises a full-length copy of the Ad2 genome from which the early region 1 genes (nucleotides 546 to 3497) have been replaced by cDNA for CFTR. The viral E1a promoter was used for CFTR cDNA; this is a low to moderate strength promoter. Termination/polyadenylation occurs at the site normally used by the E1b and protein IX transcripts. The E-3 region of the virus was conserved.

Patients

We studied three patients with CF. Genotype was determined by IG Labs (Framingham, MA). All three patients had mild CF as defined by an NIH score > 70 (Taussig et al., 1973), a normal weight to height ratio, a forced expiratory volume in 1 s (FEV1) greater than 50% of predicted and an arterial PO_2 greater than 72. All patients were seropositive for type 2 adenovirus and had no recent viral illnesses. Pretreatment cultures of nasal swabs, pharyngeal swabs, sputum, urine, stool, and blood leukocytes were negative for adenovirus. PCRs of pretreatment nasal brushings using primers for the adenovirus E1 region were negative Patients were evaluated at least twice by FEV1, cytology of nasal mucosa, visual inspection, and measurement of Vt before treatment. Prior to treatment, a coronal computed tomographic scan of the paranasal sinuses and a chest X-ray were obtained.

The first patient is a 21 year old woman who was diagnosed at 3 months after birth. She has pancreatic insufficiency, a positive sweat chloride test (101 mEq/l), and is homozygous for the ΔF508 mutation. Her NIH score was 90, and her FEV1 was 83% predicted The second patient is a 36 year old man who was diagnosed at the age of 13 when he presented with symptoms of pancreatic insufficiency. A sweat chloride test revealed a chloride concentration of 70 mEq/l. He is a heterozygote with the ΔF508 and G551D mutations. His NIH score was 88, and his FEV1 was 66% predicted. The third patient is a 50 year old woman, diagnosed at the age of 9 with a positive sweat chloride test (104 mEq/l). She has pancreatic insufficiency and insulin-dependent diabetes mellitus. She is homozygous for the ΔF508 mutation. Her NIH score was 73, and her FEV1 was 65% predicted.

Vt

The transepithelial electric potential difference across the nasal epithelium was measured using techniques similar to those previously described (Alton et al., 1987; Knowles et al., 1981). A 23 gauge subcutaneous needle connected with sterile normal saline solution to a silver/silver chloride pellet (E. W. Wright, Guilford, CT) was used as a reference electrode. The exploring electrode was a size 8 rubber catheter (modified ArgyleR Foley catheter, St Louis, MO) with one side hole at the tip. The catheter was filed with Ringer's solution containing 135 mM NaCl, 2.4 mM KH_2PO_4, 0.6 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES (titrated to pH 7.4 with NaOH) and was connected to a silver/silver chloride pellet. Voltage was measured with a voltmeter (Keithley Instruments Inc., Cleveland, OH) connected to a strip chart recorder (Servocorder, Watanabe Instruments, Japan) Prior to the measurements, the silver/silver chloride pellets were connected in series with the Ringer's solution; the pellets were changed if the recorded Vt was greater than ± 4 mV. The rubber catheter was introduced into the nostril under telescopic guidance (Hopkins Telescope, Karl Storz, Tuttlingen, Federal Republic of Germany), and the side hole of the catheter was placed next to the study area in the medial aspect of the inferior nasal turbinate. The distance from the anterior tip of the inferior turbinate and the spatial relationship with the medial turbinate, the maxillary sinus ostium, and in one patient a small polyp were used to locate the area of Ad2/CFTR-1 administration for measurements. Photographs and video recorder images were also used. Basal Vt was recorded until no changes in Vt were observed after slow intermittent 100 μl/min infusion of the Ringer's solution. Once a stable baseline was achieved, 200 μl of a Ringer's solution containing 100 μM amiloride (Merck and Co. Inc., West Point, PA) was instilled through the catheter, and changes in Vt were recorded until no further change was observed after intermittent installations. Finally, 200 μl of Ringer's solution containing 100 μM amiloride plus 10 μM terbutaline (Geigy Pharmaceuticals, Ardsley, NY) was instilled, and the changes in Vt were recorded.

Measurements of basal Vt were reproducible over time: in the three treated patients, the coefficients of variation before administration of Ad2/CFTR-1 were 3.6%, 12%, and 12%. The changes induced by terbutaline were also reproducible. In 30 measurements in nine CF patients, the terbutaline-induced changes in Vt (ΔVt) ranged from 0 mV to +4 mV; we never observed hyperpolarization of Vt. In contrast, in seven normal subjects ΔVt ranged from −1 mV to −5 mV; we always observed hyperpolarization.

Ad2/CFTR-1 Application and Cell Acquisition

The patients were taken to the operating room and monitoring was commenced using continuous EKG and pulse oximetry recording as well as automatic intermittent blood pressure measurement. After mild sedation, the nasal mucosa was anesthetized by atomizing 0.5 ml of 5% cocaine. The mucosa in the area of the inferior turbinate was then packed with cotton pledgets previously soaked in a mixture of 2 ml of 0.1% adrenaline and 8 ml of 1% tetracaine. The pledgets remained in place for 10–40 min. Using endoscopic visualization with a television monitoring system, the applicator was introduced through the nostril and positioned on the medial aspect of the inferior turbinate, at least 3 cm from its anterior tip (Figure 1B). The viral suspension was infused into the applicator through connecting catheters. The position of the applicator was monitored endoscopically to ensure that it did not move and that enough pressure was applied to prevent leakage. The virus was in contact with the nasal epithelium for a timed 30 min, the viral suspension was removed, and the applicator was withdrawn. In the third patient's right nasal cavity, the virus was applied using the modified Foley catheter used for Vt measurements. The catheter was introduced without anesthetic under endoscopic guidance until the side hole of the catheter was in contact with the area of interest in the inferior turbinate. The viral solution was infused slowly until a drop of

solution was seen with the telescope. The catheter was left in placed for 30 min and then removed

Cells were obtained from the area of virus administration approximately 2 weeks before treatment and then at weekly intervals after treatment. The inferior turbinate was packed for 10 min in cotton pledgets previously soaked in 1 ml of 5% cocaine. Under endoscopic control, the area of administration was gently brushed for 5 s. The brushed cells were dislodged in phosphate-buffered saline. Swabs of the nasal epithelia were collected, without using anesthesia, with cotton-tipped applicators. Cystospin slides were prepared and stained with Wright's stain. Light microscopy was used to assess the respiratory epithelial cells and inflammatory cells. For biopsies, sedation/anesthesia was administered as described for the application procedure. After endoscopic inspection and identification of the site to be biopsied, the submucosa was injected with 1% xylocaine, with 1/100,000 epinephrine. The area of virus application on the inferior turbinate was removed. The specimen was fixed in 4% formaldehyde and stained.

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Note Added in Proof

Since acceptance of this paper, we have been able to detect Ad2/CFTR-1-encoded CFTR transcripts in samples from the second and third patients. Although we failed to detect mRNA using our previously described reverse transcriptase-PCR techniques (Zabner et al., 1993), we were successful when we used a modified form of RNA template–specific PCR described by Shuldiner et al.: Shuldiner, A. R., Perfetti, R., and Roth, J. (1993). Meth. Mol. Biol. *15*, 1699–1716.