Cell Proliferation in Bronchial Epithelium and Submucosal Glands of Cystic Fibrosis Patients

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Integrative gene therapy typically requires dividing cells. This requirement has been perceived as an impediment for gene transfer to mature, uninjured airways where proliferation rates are very low. In diseases such as cystic fibrosis (CF) that may be candidates for integrative gene therapy, airway cell turnover is not known but may be increased as a result of chronic inflammation. To determine if cells in airway surface epithelium and submucosal glands of CF patients proliferate at an increased rate, paraffin sections of bronchial segments removed from CF patients ($n = 6$) at the time of lung transplantation or rapid autopsy and from non-CF patients ($n = 4$) undergoing lung resection or transplantation were immunostained with PC10, a monoclonal antibody to proliferating cell nuclear antigen (PCNA), a marker of proliferating cells. The PCNA index (percentage of nuclei immunostaining for PCNA) in CF bronchial surface epithelium was 17.0 ± 4.6% (mean ± SEM), substantially greater than in non-CF airways (< 0.2%). Within submucosal glands, PCNA-positive cells were more prevalent in the collecting ducts of CF patients than in those of normal subjects, but only rare mucous or serous cells were PCNA positive. These studies show that airway epithelial cell proliferation rates can be very high in inflamed CF airways. This prevalence of proliferating cells suggests that CF airway epithelium and submucosal gland ducts may be amenable to gene transfer using vectors, such as retroviruses, that require cell replication for stable integrative expression. Further studies are needed to evaluate cell proliferation in CF airways with less extensive airway injury.

A better understanding of airway epithelial cell biology is needed to optimize gene therapy for cystic fibrosis (CF) patients. Integrative gene transfer using retroviral and other vectors is more efficient when cells are replicating. The normal, mature airway epithelium in experimental animals has a very slow cell turnover and hence minimal proliferation (1, 2). However, after acute injury by inhalation of noxious agents (3, 4), epithelial cell proliferation in experimental animals increases. Limited information about airway cell proliferation in human subjects is available.

The standard procedures for identifying proliferating cells depend on incorporation of labeled nucleotides, such as tritiated thymidine or 5-bromo-2-deoxyuridine (BrdU), into DNA during the S phase of the cell cycle. These approaches are not easily applicable to human subjects because of the requirement for a "labeling period." Recently, proliferating cell nuclear antigen (PCNA), a 36-kD nuclear protein that is a cofactor for polymerase delta during replication of DNA (5, 6), has been used as a marker of proliferating cells because its expression is cell-cycle regulated, expressed during late G1 and S phases of the cell cycle but disappearing during early G2. Several monoclonal antibodies are commercially available for immunodetection of this protein in frozen and paraffin sections of a variety of tissues.

For these studies, bronchial segments of CF and non-CF patients undergoing lung transplantation, resection, or autopsy (within 3.5 h after death) were immunostained with PC10, a monoclonal antibody to PCNA, to determine proliferation rates in airway surface epithelium and submucosal glands in CF patients and in comparable regions in non-CF airways. Because CF airways are exposed to chronic and recurrent injury from infection and inflammation, we hypothesized that cells in airway surface epithelium and submucosal glands in CF patients are more proliferative than cells in non-CF airways and hence may be more amenable to gene therapy than previously thought.

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Abbreviations: 5-bromo-2-deoxyuridine, BrdU; cystic fibrosis, CF; cystic fibrosis transmembrane conductance regulator, CFTR; proliferating cell nuclear antigen, PCNA.

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Materials and Methods

Tissue Preparation

Bronchial segments were dissected from lungs immediately after surgical removal. Tissues were obtained from six CF patients (two females, four males; mean age 23 yr, range 13

TABLE 1
Human subjects

Patient	Age (*yr*)	Gender	Diagnosis	$SwCl^-$*	Genotype	Source	Airway No.†	PCNA Index (%)
Non-CF								
N1	4	F	Pulmonary hypertension	n/a	n/a	Transplant	3–5	0.2
N2	30	M	Brain death	n/a	n/a	Organ donor	2	< 0.1
N3	57	F	COPD	n/a	n/a	Transplant	3–5	< 0.1
N4	67	M	Large cell cancer	n/a	n/a	Left pneumonectomy	3–5	< 0.1
CF								
CF1	13	M	CF	107	R53X/?	Transplant	3–5	9.6
CF2	15	M	CF	112	ΔF508/ΔF508	Rapid autopsy‡	3–5	19.1
CF3	21	M	CF	88	ΔF508/1717-1G>A	Transplant	3–5	26.2
CF4	22	F	CF	100	ΔF508/ΔF508	Rapid autopsy‡	3–5	31.4
CF5	42	M	CF	119	n/a	Rapid autopsy‡	3–5	15.5
CF6	23	F	Variant CF	35§	3849+10kb C>T/?	Transplant	3–5	< 0.1

Definition of abbreviations: PCNA = proliferating cell nuclear antigen; n/a = not available; COPD = chronic obstructive pulmonary disease; CF = cystic fibrosis.
* Sweat chloride values (mmol/liter).
† Airway generation number: trachea = 1, lobar bronchus = 2, segmental bronchus = 3, and so forth.
‡ Autopsy performed immediately after death; tissue placed in fixative within 1.5 to 3.5 h after death.
§ Mean of five sweat chloride values obtained at different times.

to 42 yr) at autopsy or at the time of lung resection for lung transplantation and from four non-CF patients (two females, two males; mean age 40 yr, range 4 to 67 yr) undergoing transplantation or surgery (Table 1). The bronchial segments were intrapulmonary from airway generations 3 through 5 for all subjects except one non-CF subject (N2), a lung donor from whom the available segment was the extrapulmonary, mainstem bronchus (airway generation 2). Bronchial segments were fixed in 4% paraformaldehyde for 2 h, stored in phosphate-buffered saline with 30% sucrose for 16 h, and embedded in paraffin blocks that were identified by a code number. Positive control tissues included human tonsil (processed as above) and human fetal lung at 20 wk of gestation that was harvested within 10 min of delivery, stored in Dulbecco's minimal essential medium for 24 h, and then fixed and embedded as above.

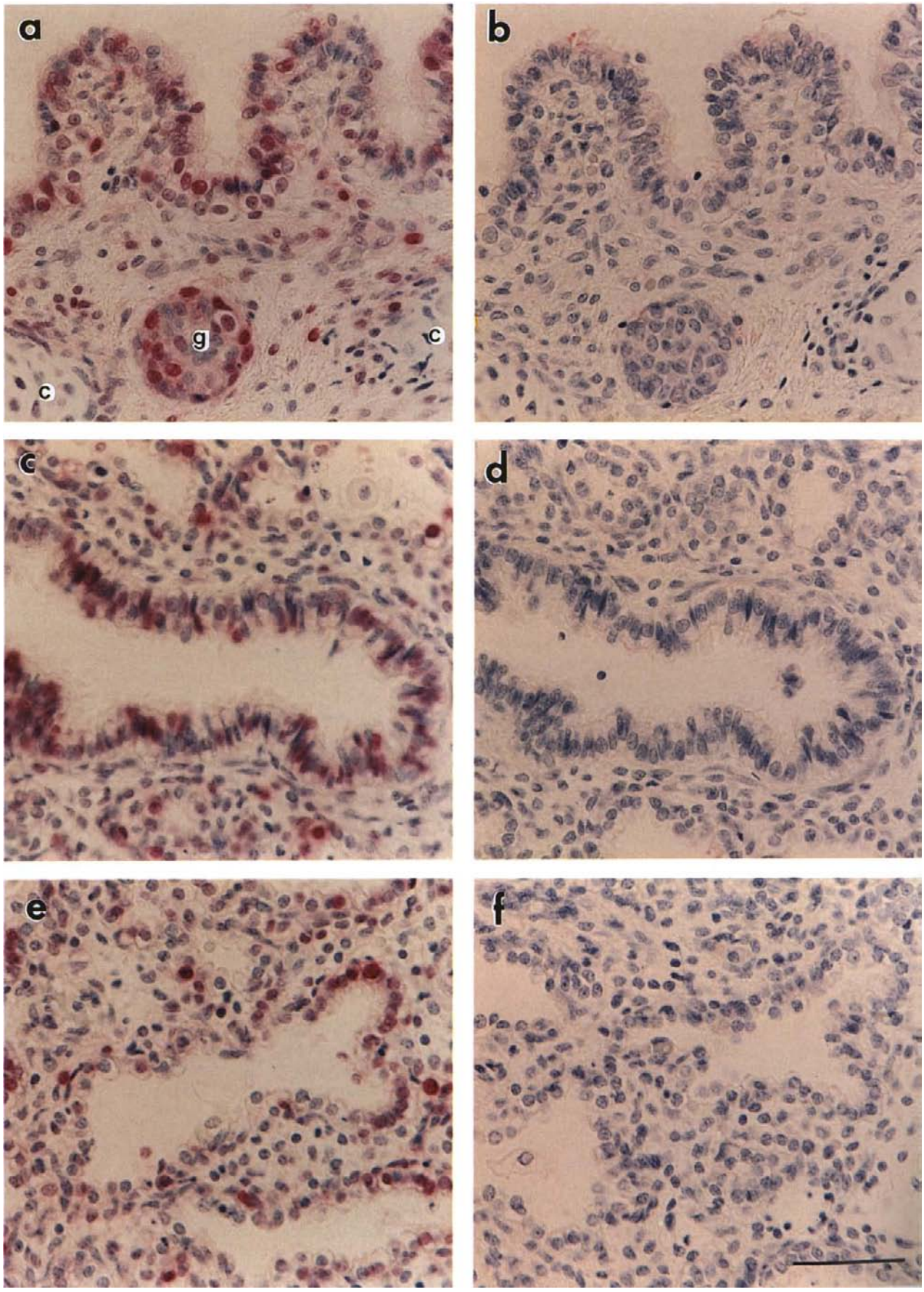
Immunohistochemistry

Bronchial cross-sections (6 μm thick) were air-dried to poly-L-lysine–coated slides and deparaffinized. PCNA immunohistochemistry was performed using the monoclonal murine antibody PC10 (IgG2a; DAKO, Carpinteria, CA) at a dilution of 1:200. Biotin-conjugated goat anti-mouse IgG (Cappel, Durham, NC) was used as the secondary antibody, and nonspecific staining was blocked by prior incubation with 0.5% normal goat serum. The ABC-alkaline phosphatase enzyme system (Vector, Burlingame, CA) with the color substrate Vector Red was used to detect bound antibody. As negative controls, adjacent sections were immunostained by the same protocol except purified mouse IgG2a was substituted for the primary antibody, PC10. Sections were counterstained with Mayer's hematoxylin and then visualized and photographed using a Nikon Microphot-FXA microscope. Sections of human tonsil (positive controls) containing a predominance of proliferating cells in their germinal centers were immunostained with each batch of airway sections to ensure technical reproducibility of staining results.

Morphologic Evaluation and Morphometry

Paraffin sections, labeled with block code number, were examined by light microscopy at 1,000× magnification without knowledge of the tissue source. At initial evaluation, the blinded examiner (M.W.L.) noted the integrity of the epithelium, presence of inflammatory cells, enlargement of submucosal glands, and dilatation of the gland duct. These gland features are characteristic of CF airways. One section per bronchial segment was morphometrically examined. All surface epithelial cell nuclei in the entire bronchial cross-section were examined and classified as PCNA positive (red nuclei from Vector Red staining) or PCNA negative (blue nuclei from hematoxylin staining). The PCNA index (percentage of PCNA-positive nuclei) was determined from counts of more than 1,000 cells around the entire circumference of each bronchial cross-section. Intraepithelial, inflammatory cell nuclei were distinguished from epithelial cell nuclei by their smaller size (approximately one-half to two-thirds the size of epithelial cell nuclei) and greater intensity of nuclear staining with hematoxylin. These intraepithelial, inflammatory cell nuclei were not included in the cell counts. The length of basal lamina was measured using an ocular grid calibrated with a micrometer slide. The contour of the basal lamina was followed as closely as possible by moving

Figure 1. Human fetal lung tissue was used as a positive control to test the PCNA immunostaining of airway epithelial cells that are proliferating during a period of rapid growth. This section from a 20-wk fetal lung demonstrates extensive proliferation of airway surface epithelial cells in bronchi (panel a), noncartilaginous airways (panel c) and distal airways (panel e), but not in negative control serial sections (panels b, d, and f, respectively) for which mouse IgG2a was substituted for the primary monoclonal antibody, PC10. In panel a, the round cluster of PCNA-staining cells (g) between two plates of cartilage (c) in the submucosal region is most likely a developing submucosal gland. Magnification is the same for these photomicrographs (bar = 50 μm).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the microscope stage and rotating the ocular grid. Submucosal gland duct openings were not included in the analyses; the line of demarcation between epithelium and gland duct was the point at which the basal lamina deviated more than 45° from the established contour. Cell density (number of nuclei per millimeter of basal lamina) was determined for each bronchial section.

Results

Histopathologic Features

The airway surface of the non-CF bronchi was lined with a pseudostratified, ciliated columnar epithelium with minimal areas (< 1% of epithelium) of disruption. In CF bronchi, most of the epithelium was pseudostratified, ciliated columnar epithelium, but there were regions of hyperplasia, squamous metaplasia, and cuboidal epithelium. The epithelium of CF bronchi was virtually continuous, with less than 5% of the surface disrupted or denuded. Regions of inflammatory cell infiltrates were apparent within the submucosa and epithelium of all CF bronchi but not in non-CF bronchi. The submucosal glands in the CF bronchi, but not non-CF bronchi, were enlarged, with dilated gland ducts often filled with mucus.

Airway Surface Epithelium

In the 20-wk-gestation fetal human lung (Figure 1), PCNA immunoreactivity was prevalent in the surface epithelium of cartilaginous, noncartilaginous, and distal airways and in the immature submucosal glands. The PCNA staining was specific. There was no PCNA staining in the adjacent negative control sections in which immunostaining was modified by substituting mouse IgG2a for the primary antibody. However, PCNA immunoreactivity in bronchi of mature, non-CF airways was extremely sparse (Figure 2).

In most of the (non-CF) bronchial cross-sections, none of the more than 1,000 surface epithelial cells were PCNA-positive, even though numerous PCNA positive cells were identified in other regions of the section, such as lymphoid tissue (Figure 2b). In most of the CF bronchial sections, airway epithelial cell proliferation was much more apparent than in non-CF controls. However, within a CF bronchial cross-section, there was regional variation (Figure 3), with areas containing rare PCNA-positive cells occurring in the same bronchial sections as areas with multiple PCNA-positive cells. One section contained a region of extensive hyperplasia in which the majority of nuclei (greater than 75%) were PCNA-positive (Figure 3c). In these preparations, the resolution was not adequate to distinguish basal cells from secretory cells; therefore, the identity of the proliferating cells could not be determined. Many of the isolated PCNA-positive nuclei (Figure 3a) were adjacent to the basement membrane; however, in regions containing clusters of PCNA-positive cells (Figures 3b and 3c), many of the PCNA-positive nuclei were not adjacent to the basement membrane and, therefore, were less likely to be typical basal cells. Infiltrating inflammatory cells, predominantly mononuclear cells, were prevalent in CF epithelial and submucosal layers. The nuclei of these infiltrating cells were easily distinguished from epithelial nuclei because of their smaller size and were not included in the cell counts.

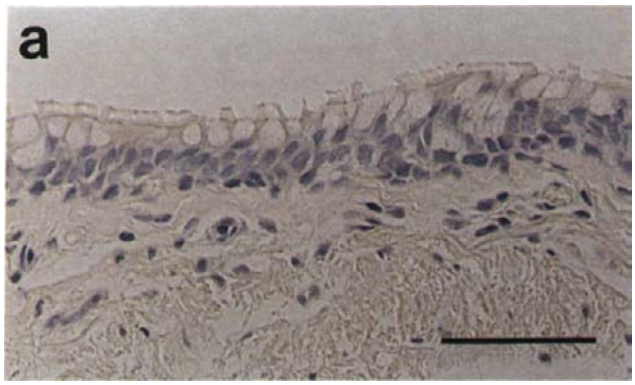
Because of the regional variation in PCNA immunoreactivity in CF bronchi, the PCNA index was determined from cell counts in the entire circumference of the bronchial cross-section to minimize sampling error. The PCNA index for the CF group (17.0 ± 4.6%, mean ± SEM) was substantially greater than for the non-CF control group (Figure 4). For three of the four non-CF control subjects, the PCNA index was less than 0.1%; none of more than 1,200 counted nuclei per cross-section was PCNA positive. The PCNA index was 0.2% in one of the four non-CF control subjects (Nl), a 4-yr-old undergoing lung transplantation for primary pulmonary hypertension. For five of the six CF patients, the PCNA indices ranged from 10 to 31%, but one CF bronchus had a PCNA index less than 0.1%. At the initial microscopic inspection of this section, the blinded examiner noted a low prevalence of PCNA-positive cells (as in normal airways) but also an inflammatory cell infiltration (predominantly mononuclear cells) and hypertrophied submucosal glands with dilated ducts. This CF patient with the low PCNA index is CF6 (Table 1), who had a variant of CF with the genotype 3849+10kb C>T and with normal sweat chloride values. Interestingly, this patient had delayed onset of lung disease (first respiratory illness noted at 5 yr of age), delayed diagnosis (at 10 yr of age), delayed acquisition of *Pseudomonas* (sputum culture at 10 yr isolated *Staphylococcus aureus* but not *Pseudomonas*), but severe, end-stage lung disease by 23 yr of age (with forced vital capacity at 26% of predicted, forced expiratory volume in 1 s at 12% of predicted, and midmaximal expiratory flow at 9% of predicted).

Despite the differences in prevalence of proliferating cells, the mean cell density in the CF bronchi was not significantly different from that in control bronchi (Figure 4); however, the cell density was more variable in the CF bronchi, most likely reflecting areas of hyperplasia and epithelial shedding.

PCNA Staining in Submucosal Glands

In non-CF submucosal glands, PCNA immunoreactivity was minimal; however, in CF glands, PCNA-staining cells were prevalent in the gland ducts and tubules but less prevalent in the acini containing mucous cells (Figure 5). Infiltrates of inflammatory cells, predominantly mononuclear cells, were apparent around CF, but not non-CF, submucosal glands.

Figure 3. In most of the CF bronchial sections, airway epithelial cell proliferation was much more apparent than in non-CF controls. In a single cross-section, regions with little PCNA staining were adjacent to areas with prevalent PCNA staining. Panels a and b reflect the variation in prevalence and intensity of PCNA staining (small arrows mark less intensely stained nuclei) as well as the marked infiltration of inflammatory cells (arrowheads) into the epithelial and submucosal layers and thickened basal lamina (BL). In the one region of extensive hyperplasia (panel c), the prevalence of PCNA-positive nuclei was greater than 75%. The negative control serial section is shown in panel d. Magnification is the same for these photomicrographs (bar = 50 μm).



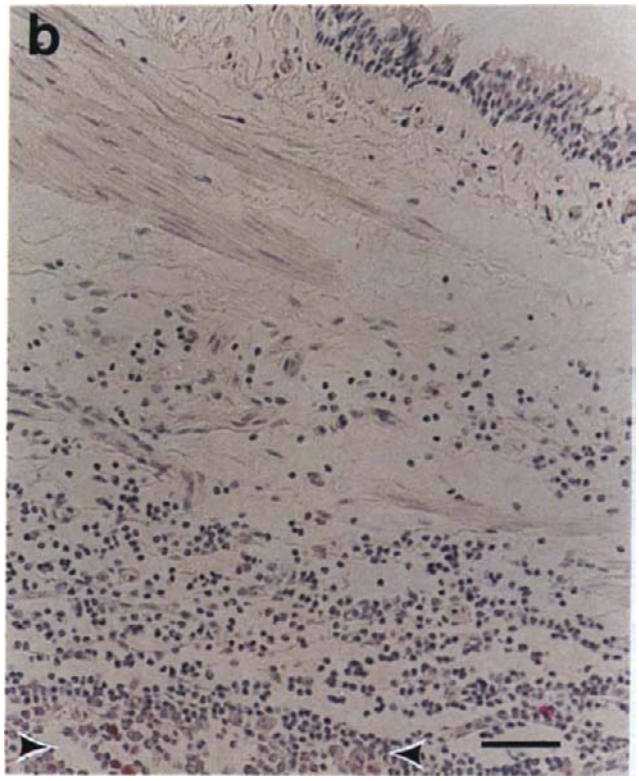
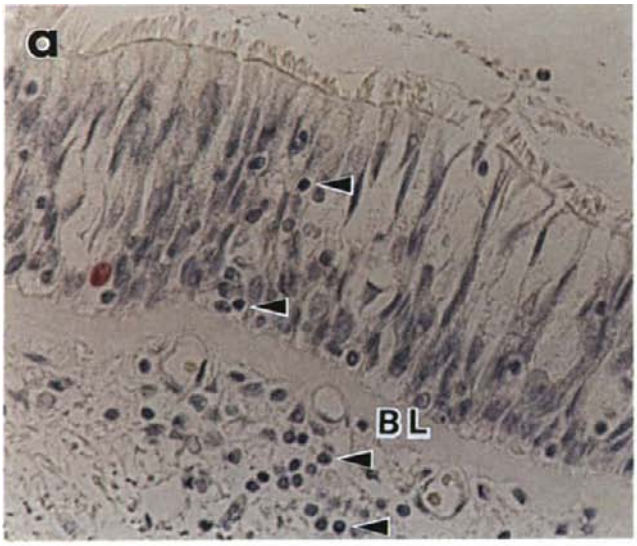
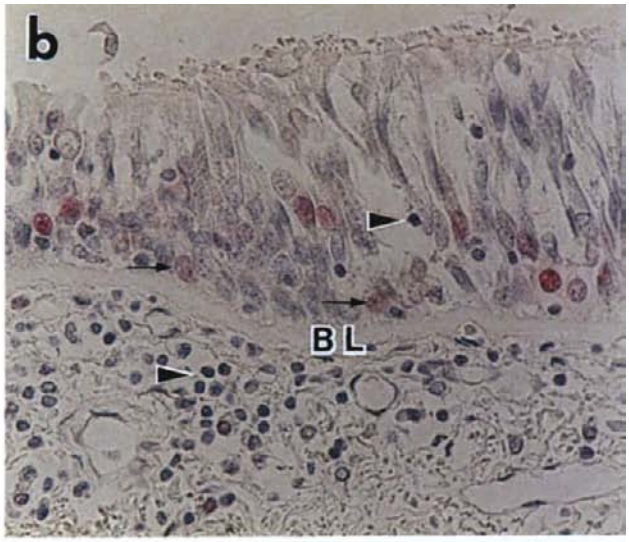
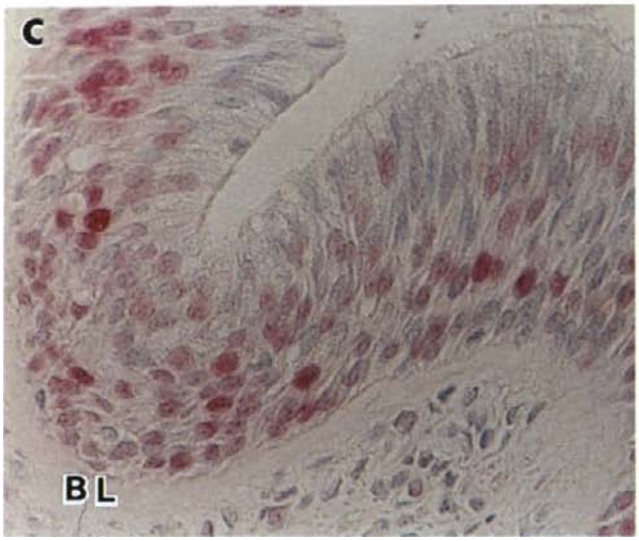
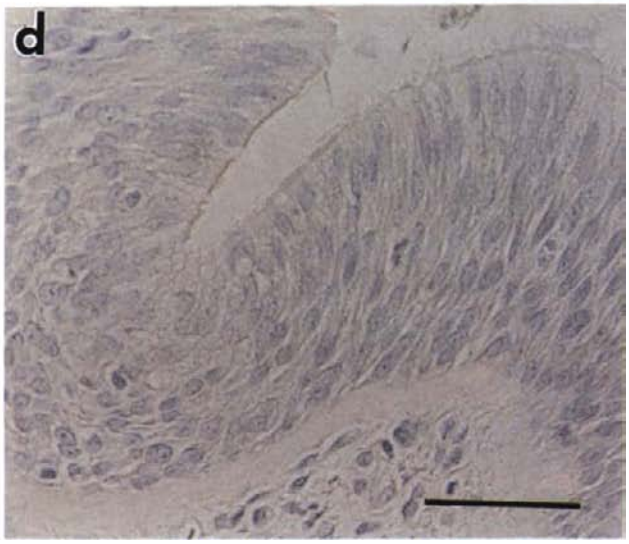


Figure 2. Epithelial cell proliferation in bronchi from non-CF lungs was extremely limited. No PCNA-staining bronchial epithelial cells were present in several cross-sections that contained more than 1,000 cells. Panel a is a representative section of a non-CF bronchus that contains no PCNA-staining cells. Panel b (at a lower magnification) demonstrates no PCNA staining in the surface epithelium but a cluster of PCNA-staining cells (between arrowheads) in the lymphoid tissue in the submucosal region. Bar = 50 μm.









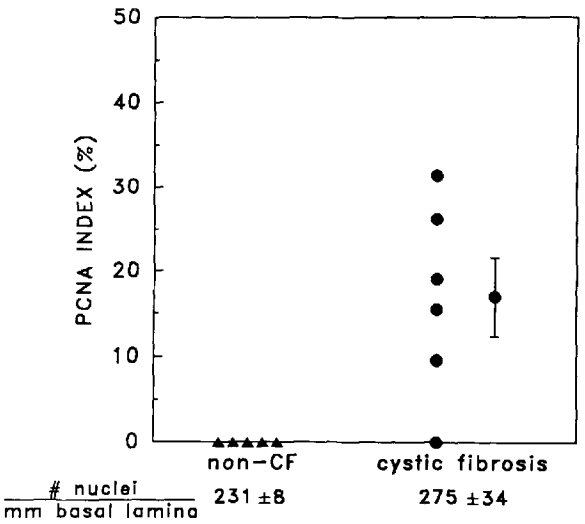


Figure 4. Cell proliferation rates in human bronchial epithelium. The PCNA index is the number of PCNA-positive nuclei divided by the total number of nuclei. More than 1,000 nuclei were counted per section. The epithelial cell density was determined from the number of nuclei per millimeter of basal lamina.

Discussion

This study uses PCNA immunostaining to assess airway epithelial cell proliferation in CF and non-CF patients. Expression of PCNA is limited to late G1 and S stages with disappearance during early G2 (7–9); therefore, presence of this antigen identifies cells that are preparing for mitosis. Most of the previous studies to identify proliferating cells in airways have used tritiated thymidine or BrdU labeling to identify cells that were in the S phase of the cell cycle at the time of labeling. Consistent with these expected differences in expression of these markers during the cell cycle, dual-stain comparisons of PCNA and BrdU labeling have demonstrated that the overlap in staining is incomplete and the proportion of cells that are PCNA positive tends to be larger than the proportion that are BrdU positive (10–12).

The major advantage of the PCNA immunostaining over BrdU or tritiated thymidine incorporation is the elimination of a "labeling period," thereby allowing application to human tissues (including archival tissues). The major disadvantage of PCNA immunostaining is the variable intensity and uniformity of PCNA immunostaining that presumably reflects differences in expression during the cell cycle (less intense in late G1 as expression is increasing and in early G2 as expression is decreasing but most intense during S phase) (7). For this study, we counted all immunostained nuclei to eliminate subjective assessment of staining intensity. Also, the immunoreactivity with the PC10 antibody may be influenced by fixation and processing conditions (13). Factors that can decrease PCNA immunoreactivity include prolonged fixation, exposure to elevated temperatures (e.g., baking sections onto slides), and types of fixative (11, 13). For this study, we limited the exposure to paraformaldehyde and to heat and included positive control sections of human tonsil to assess and ensure technical reproducibility of immunostaining.

Previous use of PCNA immunostaining in human lung has focused on assessment of cell proliferation in lung neoplasms or bronchial epithelial dysplasia (14–16). In a study of non-small cell carcinomas, the PCNA index was increased (mean 53.4%, range 0.1 to 81.4%) and correlated well with the mitotic index (number of mitoses per 1,000 cells) (15). In an analysis of normal and dysplastic bronchial epithelium in biopsies from smokers, the PCNA index in areas of mild dysplasia (median 12%, range 5 to 14%) was not different from that in nondysplastic epithelium (median 2%, range 0 to 19%) but was increased in moderate (median 37%, range 0 to 92%) and severe (median 46%, range 0 to 96%) dysplasia, suggesting that proliferation correlates with severity of dysplasia (16).

Relatively little is known about PCNA staining in nondysplastic, non-neoplastic bronchial epithelium. Preliminary analysis of bronchial biopsies showed a range of PCNA labeling in different disease states, with a mean PCNA index of 0.18% in healthy nonsmokers, 0.05% in nonsmoking asthmatics, 2.4% in smoking asthmatics, 12% in patients with chronic bronchitis, and 10.5% in patients with non-small cell lung cancer (17). These PCNA indices for healthy nonsmokers and nonsmoking asthmatics are comparable to our values for non-CF airway epithelium (< 0.1 to 0.2%), and the PCNA indices for chronic bronchitis are comparable to our values for CF airways (mean 17%, range 0.2 to 31%).

Most of the available information on proliferation of mature airway epithelium is derived from studies in rats that quantitated tritiated thymidine labeling. These studies demonstrated a low prevalence of proliferating cells in rat airway epithelium that varied with airway region (24 h labeling index of 1.0, 0.6, and 0.5% in trachea, bronchi, and bronchioles, respectively) (18). These labeling indices for normal airway epithelium are slightly higher than our non-CF PCNA indices. Possible explanations for this apparent discrepancy include the long labeling period in the rat studies (up to 24 h) and species variation. Increased airway epithelial cell proliferation has been observed after a variety of airway insults in animal models. In our study, the increase in airway epithelial cell proliferation in CF patients is comparable to that seen in animal models after infection with *Mycoplasma pulmonis* (19), mechanical trauma (20), or exposure to cigarette smoke (3), nitrogen dioxide (4, 21), or ozone (4, 22). Inflammatory cells and their products may contribute to the injury and increased cell turnover, as suggested by studies demonstrating epithelial injury and increased proliferation after exposure to neutrophil elastase (23) and studies identifying a strong relationship between epithelial necrosis and prevalence of granulocytes in airways after ozone exposure (24). Inflammatory cells, typically mononuclear cells, were prevalent in the CF airways but virtually absent in the non-CF airways examined in this study, supporting the notion that inflammatory cells and their products contribute to the increased epithelial cell turnover and resulting increased proliferation. Interestingly, the CF patient with the low PCNA index (0.2%) was a CF variant with the rare genotype 3849+10kb C>T, which is associated with normal sweat chlorides and milder lung disease than the more prevalent ΔF508 genotype (25). Inflammatory cell infiltration was apparent in airway sections of this variant CF patient, raising

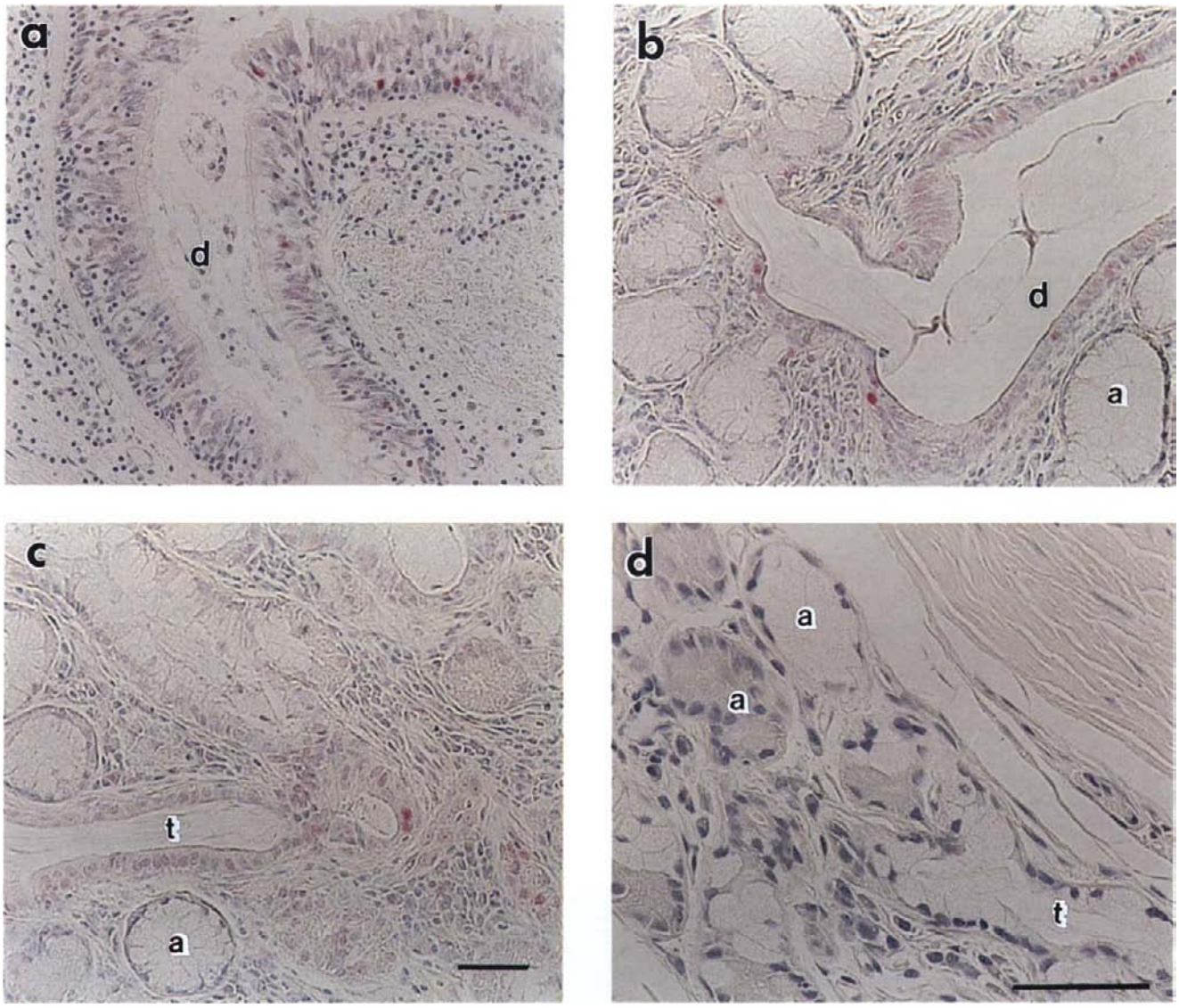


Figure 5. In submucosal glands of CF patients, PCNA-positive cells were prevalent at the duct openings shown in panel a, in dilated ducts [(d)] shown in b, and in tubules [(t)] shown in panel c, but not in the acini [(a)] of CF glands (panels a and c). In non-CF glands (panel d), PCNA-positive cells were not apparent. The magnification of photomicrographs in panels a through c is the same; however, a higher magnification is shown in panel d (bar = 50 μm).

the possibility of genetic influences on epithelial responses to inflammatory cells and their products.

A variety of factors may influence proliferation, including age, gender, and inflammatory cell influx. The small numbers of subjects limit our ability to subgroup patients to analyze the specific influence of these factors. For the control group, the youngest patient (4 yr of age) had the higher PCNA index (0.2%), suggesting that the epithelium in immature airways may be slightly more proliferative than in mature airways. For the CF group, there was no distinct age or gender relationship. As suggested above, the degree of inflammation may influence proliferation. A more definitive characterization of the relationship between inflammmation and proliferation will require morphometric analyses to quantitate prevalence of both neutrophil and PCNA-positive cells in a larger group of CF patients.

These studies demonstrating enhanced proliferation of airway epithelial cells in CF impact on strategies for gene transfer in this patient population. Approaches using integrative vectors, such as retroviruses, were initially considered to have high potential because of the likelihood of prolonged expression of the integrated transgenes but were discounted because the efficiency of these integrative vectors is limited in slowly replicating cells, as in the normal airway epithelium. The increased proliferation in inflamed CF airways provides evidence to support the use of integrative vectors in some patients. Previous studies have demonstrated that the chloride transport defect in CF airway epithelial cells can be normalized when as few as 6 to 10% of the cells in an epithelial sheet have been "corrected" by gene transfer (26). Our studies suggest that there are airway regions in CF airways in which more than 10% of the cells are proliferating and

hence susceptible to correction by vectors that are highly efficient in replicating cells. Key issues to be addressed are whether these proliferating cells are accessible to vectors that are applied to the airway lumen and whether these proliferating cells will differentiate into cells expressing cystic fibrosis transmembrane conductance regulator (CFTR). The subpopulation of cells in the submucosal glands that are proliferating (duct cells) is particularly intriguing because this is consistent with the localization of CFTR (27).

We conclude that in CF patients undergoing lung transplant because of severe lung disease, airway epithelial cells and submucosal duct cells are more proliferative than in non-CF airways. This increase in proliferation most likely reflects injury from chronic infection and inflammation. We speculate that this profuse level of proliferation should be adequate for effective gene therapy using retrovirus or adenovirus-associated virus (AAV) vectors. Further studies are needed to define cell proliferation at earlier stages of CF lung disease.

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