

# New Models of the Tracheal Airway Define the Glandular Contribution to Airway Surface Fluid and Electrolyte Composition

Xiaorong Wang, Yulong Zhang, Anson Amberson, and John F. Engelhardt

Departments of Anatomy and Cell Biology, and Internal Medicine, College of Medicine, University of Iowa, Iowa City, Iowa

Antibacterial defenses in the airway are dependent on multifactorial influences that determine the composition of both fluid and/or electrolytes at the surface of the airway and the secretory products that aid in bacterial killing and clearance. In cystic fibrosis (CF), these mechanisms of airway protection may be defective, leading to increased colonization with *Pseudomonas aeruginosa*. Submucosal glands, a predominant site of cystic fibrosis transmembrane conductance regulator (CFTR) protein expression in the airway, have been hypothesized to play an important role in protection of the airway. Furthermore, recent studies have suggested that the salt concentration at the airway surface may be a key factor in regulating the activity of antibacterial substances in the airway. To explore these issues, we have used a new model of the ferret tracheal airway to evaluate the contribution of submucosal glands in regulating airway surface fluid and electrolyte composition. Using tracheal xenograft models with and without submucosal glands, we have characterized several aspects of airway physiology that may be important in defining antibacterial properties. These endpoints included the contribution of submucosal glands in defining bioelectric properties of the surface airway epithelium, airway surface fluid (ASF) chloride composition, ASF volumes, and secretion of the antibacterial factor lysozyme. Findings from these studies demonstrate a significantly elevated secreted fluid volume ( $V_s$ ) and chloride concentration ( $[Cl]_s$ ) in ASF from airways with submucosal glands ( $V_s = 47 \pm 4 \mu\text{l}$ ;  $[Cl]_s = 128 \pm 5 \text{ mM}$ ), as compared with xenograft airways without glands ( $V_s = 36 \pm 2 \mu\text{l}$ ;  $[Cl]_s = 103 \pm 6 \text{ mM}$ ). Furthermore, a temperature labile factor secreted by submucosal glands appears to alter the baseline activation of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid and/or diphenylamine-2-carboxylic acid-sensitive chloride channels in the surface airway epithelium. Lastly, the lysozyme content of tracheal airways with submucosal glands was 8.5-fold higher than were airways without glands. These studies demonstrate that submucosal glands affect both the ionic composition and bioelectric properties of the airway and suggest that models evaluating antibacterial properties of the airway in CF should take into account the contribution of glands in airway physiology.

Cystic fibrosis (CF) is characterized by abnormal regulation of chloride transport due to defects in the gene encoding the cystic fibrosis transmembrane conductance regula-

tor (CFTR) (1). Chronic lung infections, bronchiectasis, and respiratory failure are the primary causes of mortality in CF (2). Numerous pathophysiologic mechanisms have been proposed to be involved in the progression of chronic bacterial infection in the CF lung, including improper fluid balance at the airway surface (3–5) and altered ionic composition of the airway fluid, which might create a milieu conducive to infection (6–8). Pathology in the CF lung is characterized by chronic bacterial infection in the peripheral small airways (2, 9). Hence, the small airways have been traditionally thought to be the site at which defective CFTR leads to the CF lung phenotype. However, the localization of high levels of CFTR messenger RNA (mRNA) and protein to other regions in the lung, such as submucosal glands in the conducting airways, suggests that these regions may also be involved in the pathoprogession of airway infection (10, 11). Several lines of evidence suggest that submucosal glands may be important in this disease, including the following: (1) submucosal glands appear to have defined early pathophysiology in the CF neonatal lung (12–14); (2) in the cartilaginous airways, CFTR expression is found at the highest levels in submucosal gland serous tubules (10, 11); and (3) these serous cells secrete high levels of antibacterial proteins such as lysozyme and lactoferrin (15, 16).

Two central hypotheses underlie mechanisms relating improper fluid and electrolyte balance to increased bacterial colonization in the CF airway. The most traditional view has been that increased Na absorption and decreased chloride secretion in CF leads to dehydration of the airway surface fluid (ASF) layer, which in turn leads to thick mucus with altered physical properties and impaired mucociliary clearance of inhaled bacteria (5, 17). This mechanism suggests that the innate antibacterial properties of surface airways are unaltered in CF, but the mechanical properties of clearance are impaired. In contrast, recent studies comparing the innate defense mechanisms of ASF from CF and non-CF epithelia have suggested that a higher concentration of NaCl in CF may affect the antibacterial properties of ASF (6, 17). However, this issue still remains a point of contention as others have seen little difference in the ionic composition of ASF from CF and non-CF patients (18). Such studies have led to a resurgence of investigation comparing the bactericidal and clearance properties of CF and non-CF airway epithelium in an effort to better understand the mechanisms underlying regulation of NaCl concentration in ASF and to characterize antibacterial substances in the airway that may have altered activity in CF (17, 19–22).

Several model systems have been used to functionally evaluate submucosal gland secretions and bioelectric properties. These models have included the technically challenging cannulation of single gland ducts for fluid compo-

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Address correspondence to: John F. Engelhardt, Dept. of Anatomy and Cell Biology, University of Iowa, School of Medicine, 51 Newton Rd., Rm. 1-111 BSB, Iowa City, IA 52242. E-mail: john-engelhardt@uiowa.edu

Abbreviations: airway surface fluid, ASF; cyclic adenosine monophosphate, cAMP; cystic fibrosis, CF; CF transmembrane conductance regulator, CFTR; chlorophenylthio, cpt; 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid, DIDS; diphenylamine-2-carboxylic acid, DPC; electron microscopy, EM; epithelial sodium channel, ENaC; Hepes phosphate-buffered Ringer's solution, HPBR; minimum essential medium, MEM; outward rectifying chloride channel, ORCC; potential difference, PD; surface airway epithelial, SAE; standard error of the mean, SEM; uridine triphosphate, UTP; input volume,  $V_i$ ; fluid loss,  $V_l$ ; ASF volume,  $V_s$ .

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sition analysis (23) as well as a comparison of distal porcine airways with and without submucosal glands (24). Both of these models support the importance of submucosal glands in chloride and bicarbonate secretion in the airway. Other investigators have demonstrated that the bioelectric properties change with the diameter of the airway (24–26). Although these studies provide indirect evidence for the importance of submucosal glands, which also decrease with the diameter of the airway, they are incapable of separating changes in surface airway biology and composition of channels with parallel changes in the abundance of submucosal glands. To this end, we have developed ferret xenograft models of the tracheal airway in which surface airway epithelial cells have the defined anatomic characteristics of tracheal epithelium and only the abundance of submucosal glands is altered. These airway models with and without submucosal glands can be directly used to infer the contribution of submucosal glands in regulating ASF fluid and electrolyte composition in the absence of regional differences of the surface airway epithelium.

## Materials and Methods

### Generation of Ferret Tracheal Xenografts with and without Submucosal Glands

Ferret proximal airway xenografts without submucosal glands were generated from ferret adult primary tracheal epithelial cells. In these experiments, adult ferret tracheal surface airway epithelial (SAE) cells were prepared by first rinsing ferret tracheas in Eagle's minimum essential medium (MEM) with antibiotics (50  $\mu\text{g/ml}$  ceftazidime, 50  $\mu\text{g/ml}$  colymycin, 2.5  $\mu\text{g/ml}$  fungizone, 40  $\mu\text{g/ml}$  tobramycin, 100 U/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin) for 4 h at 4°C with four to five changes. Tracheas were then filled with 0.1% protease-14 in MEM, the ends ligated closed, and incubated for 36 h at 4°C. Cells were then harvested, washed twice with 10% fetal bovine serum/MEM, and directly seeded at a density of  $1 \times 10^6$  cells into donor Fisher rat tracheas denuded of all viable epithelia by freeze thawing three times and rinsing in MEM (27, 28). Ferret xenografts were then ligated to flexible plastic tubing and transplanted subcutaneously into athymic mice as previously described (27, 28). The xenograft airway lumens remained air filled during development and were flushed every 3 d to remove accumulated mucus. These reconstituted tracheas developed a fully differentiated epithelium without submucosal glands by 4 wk after transplantation. In contrast to these reconstituted xenografts, native 5-wk-old ferret tracheas were used to model proximal airways with intact submucosal glands. Native ferret airways were generated from tracheas with a similar luminal diameter to rat tracheal xenografts with reconstituted adult SAE cells described previously. Native ferret xenografted tracheas were generated by ligating 5-wk ferret tracheas to flexible plastic tubing followed by transplantation in athymic mice.

### Morphologic Analysis of Xenografts

Xenograft airways were morphologically evaluated using several criteria. First, for light level analysis, frozen sections of xenografts fixed in 4% paraformaldehyde were used to evaluate the overall level of epithelial reconstitution and presence of submucosal glands. Second, transmission electron microscopy was used to evaluate the cellular architecture of the surface airway epithelium and submucosal glands. In these studies, xenografts were infused *in situ* with fixative (2% glutaraldehyde, 2% paraformaldehyde, 0.07 M sodium phosphate, pH 7.2), excised, and fixed in the same solution overnight at 4°C. Xenografts were then decalcified

by incubation in Cal-Rite decalcification solution (Richard Anderson Inc.) for 2 h at room temperature and then divided into three equally sized rings for transmission electron microscopy processing as previously described (29). Morphometry evaluating the percentage of ciliated, nonciliated columnar, goblet, intermediate, and basal cells in the surface airway epithelium was performed on three equally spaced quadrants obtained over the 1-cm length of each tracheal graft. In total, two independent xenografts were evaluated for each type (with or without glands) from a total of six electron microscopy (EM) samples. The following criteria were used to identify and quantitate cell types: (1) ciliated cells, as containing cilia at the apical membrane; (2) nonciliated columnar cells, as columnar cell types reaching the apical membrane with no visible cilia or mucous granules; (3) goblet cells, as columnar cell types with visible secretory granules; (4) intermediate cells, as noncolumnar triangular-shaped cells in contact with the basal lamina and rising at least one-third the height of the epithelium but not reaching the apical membrane; and (5) basal cells, as low lying electron-dense, cuboidal-shaped cells in contact with the basal lamina and containing a high nuclear-to-cytoplasmic ratio. At least 1,100 cells were quantified from six total samples from each xenograft type.

### *In Vivo* Transepithelial Potential Difference Measurements in Ferret Tracheal Xenografts

*In vivo* transepithelial potential differences (PDs) were measured in xenografts as previously described (28, 30). These methodologies have been successfully used in the human bronchial xenograft model comparing CF with non-CF airway epithelium (28, 30). Briefly, xenografts were continuously perfused with buffers through a syringe pump at 150  $\mu\text{l/min}$ . The sequence of buffers perfused typically included: (1) Hepes phosphate-buffered Ringer's solution (HPBR) containing 10 mM Hepes, pH 7.4, 145 mM NaCl, 5 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 1.2 mM Ca-gluconate, 2.4 mM  $\text{K}_2\text{HPO}_4$ , 0.4 mM  $\text{KH}_2\text{PO}_4$ , (2) HPBR with 100  $\mu\text{M}$  amiloride, (3) HPBR chloride-free (using gluconate in place of chloride), 100  $\mu\text{M}$  amiloride, (4) HPBR chloride-free, 100  $\mu\text{M}$  amiloride, 200  $\mu\text{M}$  8-cpt-cyclic adenosine monophosphate (cAMP), 10  $\mu\text{M}$  forskolin, (5) HPBR chloride-free, 100  $\mu\text{M}$  amiloride, 200  $\mu\text{M}$  8-chlorophenylthio (cpt)-cAMP, 10  $\mu\text{M}$  forskolin, 100  $\mu\text{M}$  uridine triphosphate (UTP), and (6) HPBR. Selected experiments were also performed using chloride channel blockers 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and diphenylamine-2-carboxylic acid (DPC) at 1-mM concentrations (31) and bumetanide (a basolateral  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransport blocker) at 100- $\mu\text{M}$  concentrations (32). Millivolt recordings were captured through a voltmeter and data-linked directly to a computer, which recorded millivolt measurements at 5-s intervals.

Reconstitution experiments were used to evaluate the effects of glandular secretions on the bioelectric properties of the surface airway epithelium. In these studies, secretions collected from xenograft airways with submucosal glands were subsequently used to pretreat xenografts airways without glands before functional measurements. The goal of these studies was to determine whether the decreased baseline unstimulated chloride permeability seen in xenograft airways with submucosal glands was due to inhibitory factor(s) in glandular secretions. To this end, transepithelial PDs were measured in xenograft airways without submucosal glands under the following conditions: (1) untreated for baseline measurements, (2) pretreated with 100  $\mu\text{l}$  of secretions from xenograft airways with submucosal glands for 1 h before PD measurements, or (3) pretreated with 100  $\mu\text{l}$  of boiled (10 min) secretions from xenograft airways with submucosal glands for 1 h before PD measurements. Glandular secretions used for these reconstitution experiments were collected from xenografts by perfusion with 100  $\mu\text{l}$  HPBR. The fluid was cleared from particulate

mucin by centrifugation and stored at  $-80^{\circ}\text{C}$  before use. Each xenograft airway was assayed three times (once for each condition) with a rest interval of 2 to 3 d between PD measurements.

### Chloride Content of ASF in Xenograft Airways

The content of chloride in the ASF was calculated using radioisotopic tracers as previously described (21). Fully differentiated 5-wk ferret tracheal xenografts (air filled) were flushed with 1 ml of F12 medium followed by air at 48 h before functional measurements. After 48 h of equilibration, the luminal contents were collected by flushing the xenograft airway with 100  $\mu\text{l}$  of a 5% (iso-osmotic) mannitol solution containing 20,000 total cpm of  $\text{H}^3$ -inulin. The dilution of  $\text{H}^3$ -inulin counts in the effluent was used to calculate the volume of ASF secretions ( $V_s$ ). Calculations to account for fluid loss in the trachea during sample collection ( $V_i$ ) were also taken into consideration by quantitating the amount of radioactivity in a second large wash (1 ml) of the xenograft lumen after sample collection, as previously described (21). Formulas for calculating the ASF volume ( $V_s$ ) were based on the following equation:  $V_s = V_e - V_i + V_1$ , where the input volume ( $V_i$ ) was equal to 100  $\mu\text{l}$  and the effluent volume ( $V_e$ ) was equal to  $\{([\text{inulin}]_e/[\text{inulin}]_i) \times (V_i - V_1)\}$  (21).

The chloride content of ASF ( $[\text{Cl}]_s$ ) was directly measured on duplicate 10- $\mu\text{l}$  samples using a solid-state, chloride-specific electrode and calculated against chloride standards in 5% mannitol. The linear range of these calculations was from 1 to 200 mM NaCl. Formulas for calculating the ASF chloride concentrations were based on the following equation:  $[\text{Cl}]_s = \{([\text{Cl}]_e \times (V_s + V_i - V_1))\}/V_s$ , where the concentration of chloride in the effluent ( $[\text{Cl}]_e$ ) was empirically measured (21).

### Western Blot Analysis of Lysozyme Content in ASF

The lysozyme content of xenograft ASF secretions collected as described previously was evaluated by Western blot analysis using antilysozyme antibodies (Chemicon International Inc., Temecula, CA). In brief, 10  $\mu\text{l}$  (approximately one-fourth of secretory volume) of ASF was denatured by boiling in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, electrophoresed on a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antilysozyme antibody. Immunoreactivity was detected with a peroxidase-conjugated secondary antibody and chemiluminescence detection on X-ray film. The content of lysozyme in the secretions of xenografts with ( $n = 13$ ) and without ( $n = 13$ ) submucosal glands was compared by densitometry of Western blots.

### Statistical Methods

Comparisons between experimental data from xenografts with and without submucosal glands were performed using the two-tailed Student's *t* test. *P* values of  $< 0.05$  were considered to demonstrate significant differences between data sets.

## Results

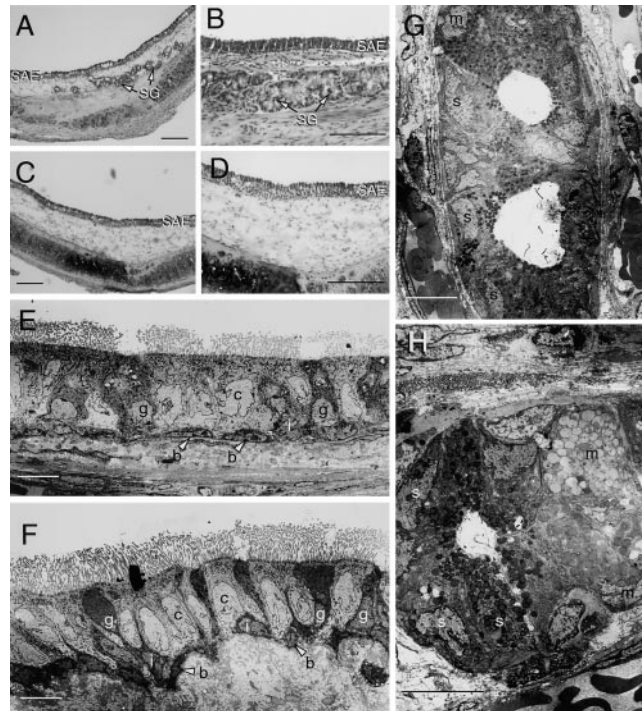
### Generation of Ferret Tracheal Xenografts with and without Submucosal Glands

By expanding progenitor cell populations with a pluripotent capacity for airway surface epithelial differentiation but limited capacity for submucosal gland development, we have developed a xenograft method for generating fully differentiated ferret proximal airways without submucosal glands (Figures 1C and 1D). In contrast, native airway xenografts generated from 5-wk-old ferret tracheas contain a fully differentiated surface airway epithelium and submucosal glands (Figures 1A and 1B). Submucosal glands of

5-wk-old ferret tracheal xenografts contain both serous and mucous tubules, markers of gland differentiation (Figures 1G and 1H). To assess whether the surface airway epithelium of reconstituted and native tracheal xenografts had similar cellular compositions, morphometry was performed on electron micrographs of each xenograft type. In these studies, the percentages of ciliated, nonciliated columnar, goblet, intermediate, and basal cells were evaluated in two independent xenografts of each type (Figures 1E and 1F). Results from these studies, which evaluated at least 1,100 cells from each type of xenograft, are presented in Table 1. No significant differences in the cellular composition of airway xenografts with and without submucosal glands were observed.

### Lysozyme Production Is Significantly Greater in Xenograft Airways with Submucosal Glands

As an indicator of submucosal gland function in native ferret xenografts, we assessed the level of lysozyme expres-



**Figure 1.** Ferret proximal airway model with and without submucosal glands. Native 5-wk ferret tracheal xenografts were transplanted subcutaneously in the flanks of nu/nu mice and harvested 3 wk post-transplantation (A, B, E, G, and H). In contrast, ferret proximal airway xenografts without submucosal glands were generated from adult primary airway epithelial cells seeded onto denuded rat tracheas (C, D, and F). Panels A through D represent light level photomicrographs stained in hematoxylin and eosin. Note the abundance of fully differentiated submucosal glands (SG) only in native tracheal xenografts (A and B). Panels E through H represent transmission electron photomicrographs of surface airway epithelium (SAE) (E and F) and submucosal gland tubules (G and H). Labels are marked as follows: c = ciliated cell; g = goblet cell; i = intermediate cell; b = basal cell; s = serous cell; m = mucous cell. Micron bars in panels A through D are 200  $\mu\text{m}$  and in panels E through H are 25  $\mu\text{m}$ .

TABLE 1  
Quantification of surface airway epithelial cell types

Xenograft Type	Ciliated Cells (%)	Goblet Cells (%)	Nonciliated Columnar Cells (%)	Intermediate Cells (%)	Basal Cells (%)
Without glands	39.4 ± 3.3	13.7 ± 5.7	5.3 ± 2.3	14.6 ± 3.5	27.1 ± 3.6
With glands	41.4 ± 2.2	13.9 ± 2.6	4.1 ± 2.4	14.3 ± 2.8	26.4 ± 3.3

Values represent the mean ± SEM from  $n = 6$  independent EM samples for each xenograft type (three samples from each of two xenografts). Approximately 150 to 250 cells were quantified from photomicrographs of each EM sample with a total of at least 1,100 cells for each xenograft type.

sion in ASF from xenografts with submucosal glands compared with the levels in reconstituted xenografts without glands. Lysozyme is an antibacterial enzyme known to be abundantly produced by serous cells of submucosal glands. In these Western blot studies, lysozyme levels were 8.5-fold higher ( $P < 0.001$ ,  $n = 13$ ) in ASF from the xenografts with submucosal glands (Figure 2). At present, it is unclear whether cross-reactivity of our antibodies with mouse lysozyme may contribute to a low background level of lysozyme in both types of xenografts. However, these results demonstrate that submucosal glands contribute more lysozyme to the ASF than the surface airway epithelium does.

#### In Vivo Transepithelial PD Measurements in Ferret Tracheal Xenografts

Bioelectric properties of xenograft airways with and without submucosal glands were evaluated using *in vivo* transepithelial PD measurements to gain insight into ion transport differences between these two structurally distinct airways.

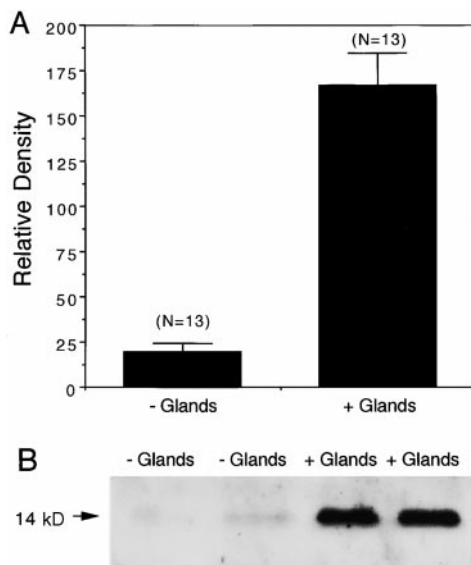


Figure 2. Lysozyme production from ferret tracheal xenografts. The level of lysozyme in secretions harvested from ferret xenografts with and without submucosal glands was quantified by Western blot. Western blots were performed with 13 ASF samples harvested from 13 independent xenografts of each type, and the intensity of the 14-KD lysozyme band was quantified by densitometry. (A) The mean ± SEM ( $n = 13$ ) relative density of lysozyme from these experiments. (B) A Western blot of two representative ASF samples harvested from xenografts with (+ glands) and without (- glands) submucosal glands.

Typical tracings from airways with and without submucosal glands are demonstrated in Figures 3A and 3B, respectively. A summary of bioelectric properties for greater than 28 xenografts of each type (Figure 3C) present several findings supporting the hypothesis that submucosal glands affect the overall ion transport properties of the airway. These findings included: (1) a significantly higher  $\Delta mV$  PD in response to amiloride in xenografts with submucosal glands ( $P < 0.002$ ); (2) a significantly higher  $\Delta mV$  PD in response to a Cl-free buffer without a cAMP agonist in airways without submucosal glands ( $P < 0.001$ ); and (3) a significantly higher cAMP/forskolin and UTP  $\Delta mV$  PD response in airways with submucosal glands ( $P < 0.001$ ).

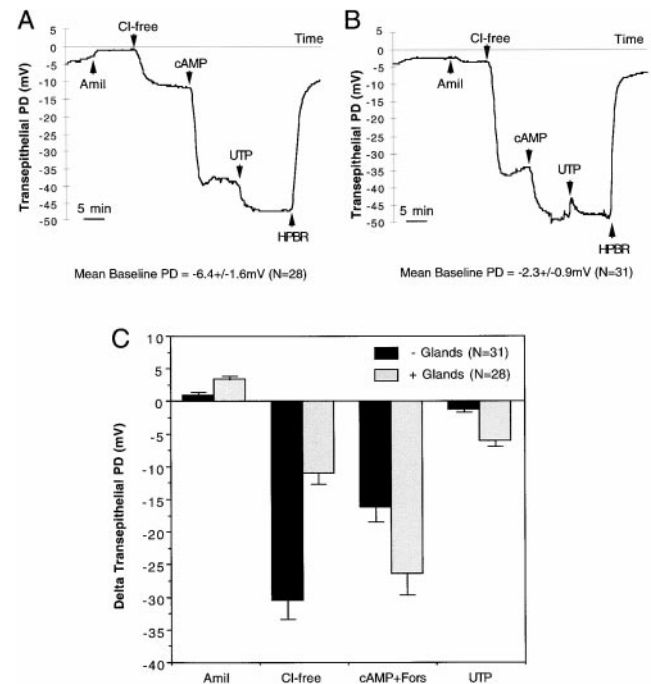
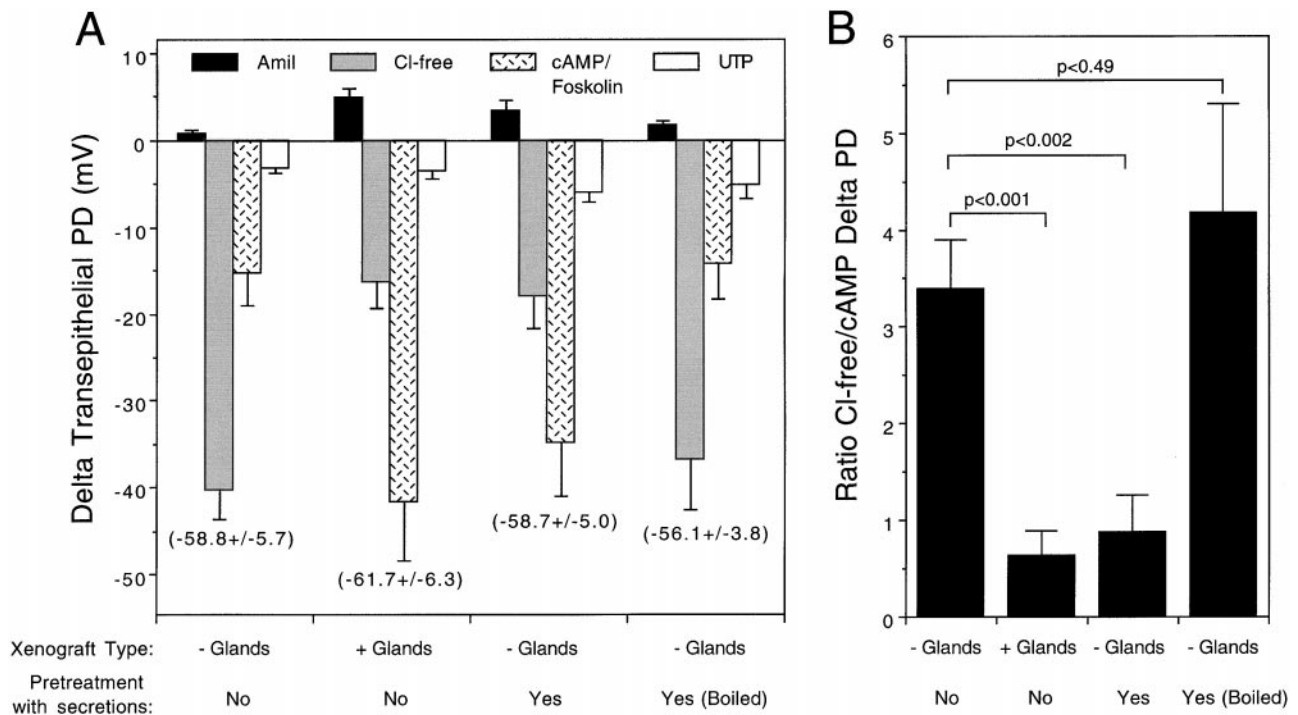


Figure 3. Transepithelial PD measurements of ferret tracheal xenografts with and without submucosal glands. Representative PDs from ferret tracheal xenografts with submucosal glands (A) and without submucosal glands (B) are shown. The following sequential buffer changes were used: (1) HPBR, (2) HPBR, 100  $\mu M$  amiloride, (3) HPBR (Cl-free), 100  $\mu M$  amiloride, (4) HPBR (Cl-free), 100  $\mu M$  amiloride, 200  $\mu M$  8-cpt-cAMP, 10  $\mu M$  forskolin, (5) HPBR (Cl-free), 100  $\mu M$  amiloride, 200  $\mu M$  8-cpt-cAMP, 10  $\mu M$  forskolin, 100  $\mu M$  UTP, and (6) HPBR. (C) Summary of the  $\Delta mV$  changes in response to amiloride, Cl-free buffer, cAMP/forskolin, and UTP. Results show the mean ± SEM for  $n$  independent measurements.

Despite these functional differences in bioelectric properties, the total  $\Delta$ mV PD of combined Cl-free/cAMP/forskolin/UTP induced changes in xenografts without submucosal glands ( $-47.9 \pm 3.0$  mV,  $n = 31$ ) was not significantly different ( $P < 0.225$ ) from xenograft airways with submucosal glands ( $-42.0 \pm 3.7$  mV,  $n = 28$ ). We were intrigued by the finding that airways without submucosal glands had a significantly higher baseline activated Cl permeability (i.e., in the absence of cAMP agonists) than did airways with submucosal glands. Such a finding suggests that the presence of submucosal glands may suppress the baseline activation of surface airway epithelial Cl channels.

To test the hypothesis that glandular secretions inhibit the baseline activation of chloride channels in the surface airway epithelium, glandular secretions were collected from native xenografts with glands and used to pretreat reconstituted xenografts without glands before transepithelial PD measurements. Results shown in Figure 4 clearly demonstrate that the baseline chloride permeability of glandless xenografts is decreased by a 1-h pretreatment with glandular secretions. In contrast, boiling of glandular secretions before pretreatment abolished the functional effect on baseline chloride permeability of airways without submucosal glands (Figure 4A). As additional negative controls,

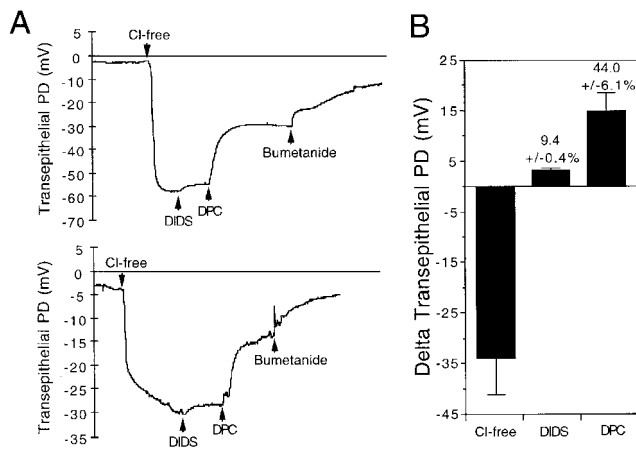
two supplementary experimental manipulations were performed, which included: (1) treatment of glandless xenografts with fluid collected from the same xenograft type before PD measurements and (2) treatment of xenografts with glands with fluid collected from xenografts without glands before PD measurements. In both these cases, no significant differences in the PD profiles of the respective xenograft type were observed after exposure to secretions (data not shown). These results demonstrate that the phenotypic bioelectric properties of airways without submucosal glands can be altered to appear like airways with submucosal glands through the action of a compound secreted by glands. This finding can be best depicted by comparing the ratio of Cl-free to cAMP/forskolin  $\Delta$ PDs, as shown in Figure 4B. In this comparison, the PD ratios of glandless airways were significantly altered after treatment with submucosal gland secretions ( $P < 0.002$ ). In contrast, no significant difference in this ratio was seen in glandless airways after treatment with boiled secretions. Furthermore, in all xenografts, the increased baseline chloride permeability in the presence of Cl-free solutions was correlated with a decreased amiloride-sensitive change in the PD. These bioelectric data also suggest that amiloride-sensitive surface airway epithelial Na channels may be ac-



**Figure 4.** Submucosal gland secretions alter the baseline chloride permeability of the SAE. To test whether submucosal gland secretions alter the baseline chloride permeability of the surface airway epithelium, secretions were collected from xenografts with submucosal glands and used to pretreat xenograft airways without submucosal glands before measurements of the bioelectric properties. An additional parameter involved pretreatment with boiled (10 min) submucosal gland secretions. Results in (A) depict the changes in transepithelial PD in response to sequential treatment with the following buffers: (1) HPBR, (2) HPBR, 100  $\mu$ M amiloride, (3) HPBR (Cl-free), 100  $\mu$ M amiloride, (4) HPBR (Cl-free), 100  $\mu$ M amiloride, 200  $\mu$ M 8-cpt-cAMP, 10  $\mu$ M forskolin, and (5) HPBR (Cl-free), 100  $\mu$ M amiloride, 200  $\mu$ M 8-cpt-cAMP, 10  $\mu$ M forskolin, 100  $\mu$ M UTP. The type of xenograft and pretreatment condition is indicated below the x-axis. Pretreated xenografts were infused with submucosal gland secretions for 1 h before functional measurements. Values represent the mean  $\pm$  SEM  $\Delta$ PD for seven independent measurements each on independent xenografts. (B) The ratio of Cl-free to cAMP/forskolin  $\Delta$ PD for each of the conditions. Values represent the mean  $\pm$  SEM  $\Delta$ PD ratio for the seven independent measurements of xenografts shown in A. Statistical comparisons were performed using an unpaired Student's *t* test with *P* value indicated above the brackets.

tivated by submucosal gland secretions directly or perhaps through alterations in the activity of other associated ion channels. Given the previously described phenomenon that activated CFTR inhibits epithelial sodium channel (ENaC) activity (4), these findings may implicate CFTR in the functional pathway leading to high levels of baseline, unstimulated chloride permeability in xenografts without submucosal glands. If this were the case, one might hypothesize that submucosal gland secretions inhibit the baseline activation of CFTR in the surface airway epithelium, leading to increased ENaC activity. To determine which potential chloride channel(s) might be activated solely by a chloride chemical gradient in xenograft airways without submucosal glands, we evaluated the pharmacologic inhibitory profile of the transepithelial PD under Cl-free conditions.

Several candidate chloride channels, including Ca<sup>+</sup>-activated Cl channels, CFTR, and/or the outward rectifying chloride channel (ORCC), could be responsible for the higher level of baseline chloride permeability in xenograft airways without submucosal glands. Both the Ca<sup>+</sup>-activated Cl channel and ORCC are inhibited by the chloride channel blocker DIDS but not by DPC (31). In contrast, CFTR is inhibited by DPC but not by DIDS (31). Results presented in Figure 5 demonstrate that DIDS only partially ( $9.4 \pm 0.4\%$ ) inhibited the Cl-free  $\Delta$ PD of reconstituted xenografts without submucosal glands. In contrast, DPC inhibited  $44.0 \pm 6.1\%$  of the Cl-free response. The additive inhibition of Cl-free induced chloride permeabil-



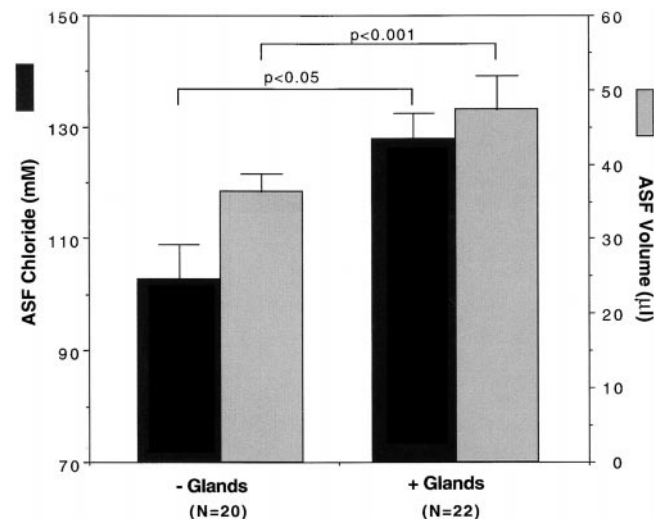
**Figure 5.** Multiple chloride channels contribute to increased baseline chloride permeability in xenograft airways without submucosal glands. The inhibitory profile of the baseline, unstimulated chloride permeability was evaluated in four independent xenografts without submucosal glands after sequential treatment with 1 mM DIDS, 1 mM DPC, and 100  $\mu$ M bumetanide. (A) The profiles for two of the xenografts analyzed. Xenografts were perfused with the following order of buffers: (1) HPBR, 100  $\mu$ M amiloride, (2) HPBR (Cl-free), 100  $\mu$ M amiloride, (3) HPBR (Cl-free), 100  $\mu$ M amiloride, 1 mM DIDS, (4) HPBR (Cl-free), 100  $\mu$ M amiloride, 1 mM DIDS, 1 mM DPC, and (5) HPBR (Cl-free), 100  $\mu$ M amiloride, 1 mM DIDS, 1 mM DPC, 100  $\mu$ M bumetanide. Arrows indicate the position of buffer changes. (B) The mean  $\pm$  SEM ( $n = 4$ )  $\Delta$ PD after Cl-free, DIDS, and DPC treatments. Values at the top of DIDS and DPC bars indicate the mean  $\pm$  SEM ( $n = 4$ ) percent inhibition of the Cl-free response and were calculated from the percentages of each individual xenograft.

ity by both DIDS and DPC suggests that multiple chloride channels in the surface airway epithelium are involved in this electrogenic pathway (i.e., Ca<sup>+</sup>-activated Cl channels, CFTR, and/or ORCC). The baseline activity of these channels appears to be elevated in airways without submucosal glands and inhibited by glandular secretions.

### Electrolyte Composition of ASF in Xenografts with and without Submucosal Glands

Using a sensitive method for calculating  $V_s$  and  $[Cl]_s$ , we sought to directly evaluate whether the presence of submucosal glands affected the steady-state concentration of chloride in the airway. The results from these comparisons are shown in Figure 6. Of note is the fact that ASF volumes from ferret airway xenografts without submucosal glands ( $36 \pm 2 \mu$ l) were significantly ( $P < 0.05$ ) lower than those in tracheal airways with submucosal glands ( $47 \pm 4 \mu$ l). Furthermore, ASF volumes in reconstituted ferret xenograft airways were extremely similar to human bronchial xenografts without submucosal glands ( $35 \mu$ l) (21). These findings suggest that the ferret airway xenografts closely mimic human airway epithelium with respect to fluid transport properties. However, most important to the present study, these findings directly demonstrate that submucosal glands influence the steady-state volume of fluid and secretions in the airway lumen of this xenograft model.

Ferret tracheal airways with and without submucosal glands also demonstrated significant differences ( $P < 0.001$ ) in the ASF chloride content (Figure 6). Ferret xenograft airways with submucosal glands had a mean chloride concentration of  $128 \pm 5$  mM, whereas the chloride content in airways without submucosal glands was  $103 \pm 6$  mM. These data demonstrate that submucosal glands not only contrib-



**Figure 6.** ASF volume and chloride concentrations in ferret tracheal xenografts with and without submucosal glands. Xenograft ASF volumes ( $V_s$ ) and chloride concentration ( $[Cl]_s$ ) were calculated using H<sup>3</sup>-inulin as an internal dilutional marker while harvesting ASF in iso-osmotic mannitol. Results show the mean  $\pm$  SEM for  $n$  independent measurements, and  $P$  values as determined by the Student's  $t$  test are indicated.

ute to the volume but also contribute significantly to the chloride content of ASF.

## Discussion

The present studies evaluating the contribution of submucosal glands in regulating the composition of ASF have used novel xenograft models, allowing direct comparisons of fully differentiated airways with and without submucosal glands. The advantages of this model system include the fact that grafted airways are vascularized and air-exposed, yet easily accessible for functional studies. *In vitro* studies evaluating polarized monolayers cannot take into account the contribution of glandular structures, whereas the present xenograft models can. Furthermore, morphometric analyses confirmed that the cytoarchitecture of the surface epithelium of gland-containing and glandless xenograft airways was not significantly different. Thus, these models provide a superior system for dissecting the relative contribution of submucosal glands to airway physiology.

Evaluation of the bioelectric properties of glandular versus nonglandular ferret tracheal airways demonstrated several significant differences with respect to anion permeability. Most notably, the ratio of Cl-free to cAMP-stimulated changes in transepithelial PD is significantly different between airways with and without glands. In the absence of submucosal glands, higher baseline active chloride permeability in the presence of low luminal chloride suggests glands may inhibit the activation state of surface airway epithelial chloride channels. Pretreatment of glandless airways with secretions harvested from xenografts with submucosal glands reversed the ratio of low chloride to cAMP-inducible transepithelial PD, providing support for this hypothesis. These effects were not the result of changes in the overall capacity of airways to transport chloride, as the total  $\Delta$ PD in the presence of low chloride, cAMP, and UTP remained relatively constant before and after conditioning of grafts with secretions ( $-58.7 \pm 5.0$  mV for grafts treated with secretions and  $-56.2 \pm 3.8$  mV for untreated grafts). Such a hypothesis, that glandular secretions alter the bioelectric properties of the surface airway epithelium, is not unreasonable, given the fact that glands have been shown to secrete adenosine triphosphate, phosphatases, ecto-adenosine triphosphatases, and other molecules that may inhibit or activate surface airway epithelial receptors controlling chloride channels (33, 34). Alternatively, submucosal glands might invoke the secretion of some factor from the surface airway epithelium in xenografts with submucosal glands, which is responsible for inhibiting the baseline activation of chloride channels in xenografts without glands. Experiments demonstrating that boiled glandular secretions lose their capacity to alter the baseline chloride permeability of airway epithelium without glands suggest that the effector molecule is heat labile and hence might be an enzyme. Multiple types of channels appear to be responsible for the higher chloride permeability in airways without glands because they are inhibited by both DIDS and DPC. However, the majority of the response appears to be primarily sensitive to DPC and could in fact be attributable to CFTR.

Differences in bioelectric properties of xenograft airways with and without submucosal glands were mirrored

by differences in ASF chloride content. ASF chloride from glandular airways was significantly higher than that in airways without submucosal glands. Several hypotheses might account for these differences. First, as previously demonstrated by our laboratory (21), mucin interferes to some extent with chloride determination measurements. High concentrations of mucin in ASF from xenografts with submucosal glands could lead to an overestimation (12% at 0.5 mg/ml mucin) of the chloride concentration. Although it is difficult to assess differences in the free mucin content of ASF because much is precipitated in aggregates, this alone is unlikely to account for the 24% higher level of chloride in ASF from xenografts with submucosal glands. Second, submucosal glands may secrete chloride-rich fluid into the airway. Given the fact that CFTR is highly abundant in submucosal glands, this hypothesis seems reasonable. In contrast, others have suggested that glandular secretions from human nasal epithelium are hypotonic. However, these studies were performed under conditions of stimulated glandular secretion (18), whereas the present study evaluated baseline, unstimulated conditions. Third, the higher baseline activation of chloride channels in xenograft airways without submucosal glands suggests an alternative hypothesis that glandular secretions inhibit absorption of NaCl in the airway by modulating chloride permeability. One point in question regarding this alternative hypothesis pertains to the pathways for sodium absorption in the ferret airway. Amiloride-sensitive sodium permeability is significantly lower in ferret airways without submucosal glands. Treatment of these glandless airways with secretions harvested from xenografts with glands was capable of increasing amiloride-sensitive sodium permeability 4-fold, to near the level seen in native xenograft airways with submucosal glands. Furthermore, changes in amiloride-sensitive sodium permeability produced by glandular secretions appeared to inversely mirror changes in the level of unstimulated chloride permeability (i.e., increases in amiloride-sensitive  $\Delta$ PD correlated with a reduction in low chloride  $\Delta$ PD). Given the fact that this low chloride response was inhibited 44% by DPC, our results are consistent with CFTR suppression of ENaC channels in the surface airway epithelium. However, differences in the amiloride-sensitive sodium permeability in airways with and without glands might also be due in part to abundant sodium channels in submucosal gland ducts. In fact, recent investigations have localized high levels of mRNAs for all three of the ENaC subunits in submucosal gland ducts (35). At face value, increased ENaC activity and decreased baseline chloride permeability in xenografts with submucosal glands would be anticipated to activate fluid absorption and decrease ASF volume (i.e., as is seen in CF airways). However, this was not the case. Hence, one would conclude that although the inverse link between sodium and chloride permeability is interesting, it does not likely account for differences in ASF volumes.

In conclusion, our results demonstrate that the presence of submucosal glands in the airway significantly alters the bioelectric properties, the steady-state chloride content, and the volume of airway secretions. One of the most interesting and novel findings includes the fact that a heat-labile factor secreted by submucosal glands alters the ac-

tivity of chloride channels (perhaps including CFTR) in the surface airway epithelium. These findings suggest that the properties of ion and fluid transport in proximal airways are controlled by a dynamic interaction between submucosal glands and the surface airway epithelium. The relevance of these findings becomes important as the field continues to deliberate on the importance of ASF composition in the protection of the airway from infection. Several abundant antibacterial compounds in the airway, including lysozyme and  $\beta$ -defensin, have been shown to be sensitive to the higher salt concentration in ASF from CF patients (8, 36, 37). Concentrations of chloride in ASF from both xenografts with and without submucosal glands are within the range of reported salt concentrations that begin to inhibit defensin antibacterial properties. However, the antibacterial properties of the airway are likely controlled by multifactorial influences determining both the composition and viscoelastic properties of the fluid that lines the airways. Our data demonstrating that submucosal glands alter the bioelectric properties of the surface airway epithelium suggest that models used to dissect these pathophysiologic mechanisms should take into account glandular function in the airway.

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