Absence of Secretion to Vasoactive Intestinal Peptide in Cystic Fibrosis Airway Glands*

We are testing the hypothesis that the malfunctioning of airway gland serous cells is a component of cystic fibrosis (CF) airway disease. CF is caused by mutations that disrupt CF transmembrane conductance regulator, an anion channel essential for proper fluid secretion in some epithelia. Submucosal glands supply most of the mucus in upper airways, and gland serous cells are the primary site of CF transmembrane conductance regulator expression in airways. We have discovered a major defect in CF glands by in situ optical monitoring of secretions from single human airway glands. CF glands did not secrete to agents that elevated [cAMP]i (0 responses/450 glands, 8 subjects), whereas glands were responsive to all donor tracheas (605/827 glands, 15 subjects) and in bronchi from subjects who were transplanted because of other lung diseases (148/166 glands, n = 10). CF glands secreted to cholinergic stimulation, and serous cells were abundant in glands from all CF subjects. The complete absence of secretion to agents that elevate [cAMP]i suggests that altered secretion of gland mucus could contribute to CF lung disease.

Cystic fibrosis is characterized by widespread dysfunction of exocrine organs (1). Organs that secrete mucus or macromolecules, including the sinuses, vas deferens, pancreas, and intestine, become partially or completely filled with inspissated secretions, often before or shortly after birth, leading in some cases to complete blockage and degeneration (2–4). Inadequate hydration of epithelial fluids underlies much of this pathology. The proposed role of salt and water was made even before the discovery that CF1 is caused by mutations in CFTR, an anion channel and channel regulator essential for proper salt and water movement across some epithelia (5). There is increasing evidence that this general dysfunction also plays an important role in CF airway disease (6).

The most devastating clinical consequence of CF is chronic infection of airways with normally innocuous bacteria and fungi. There is as yet no consensus on how this is related to altered epithelial salt and water transport, but the earliest and most persistent hypothesis has been that some defect in airway mucus is responsible. The role of mucous clearance as a primary innate defense mechanism of the airways has recently received renewed attention (6). In CF airways, infecting organisms are confined to the mucus (6–9) (for discussion, see Ref. 10), but they are neither killed nor cleared, and their diffusible products provoke an intense but ineffective neutrophilic inflammatory response that further degrades the airways, eventually leading to death from pulmonary failure (11).

Within airways, CFTR is most highly expressed in serous cells of submucosal glands (12). Submucosal glands, which are estimated to supply >95% of upper airway mucus (13), occur in bronchi from subjects who were transplanted because of other lung diseases (148/166 glands), n = 10. Normal glands are ~60% serous and 40% mucous cells by volume, and the abundant serous cells secrete water, electrolytes, and a rich mixture of antimicrobial, anti-inflammatory and antioxidant substances, whereas mucous cells provide most of the mucin component (14, 15). Because of their key role in fighting mucosal infections, serous cells have been described as “immobilized neutrophils” (16).

Remarkably, despite their possible relevance for CF lung disease, the behavior of intact, CF submucosal glands has never been directly studied. However, ion and fluid secretion is reduced in cultures of CF gland cells (18, 19) and in glands pharmacologically treated with CFTR inhibitors (20). This is consistent with patch clamp studies of primary cultures of serous cells (21) and the Calu-3 serous cell model (22), which indicate that CFTR is the only physiologically relevant apical anion channel in such cells. Ussing chamber studies with permeabilized cell sheets of Calu-3 cells confirm those studies and further indicate that functional CFTR is required for secretion to calcium-elevating agonists (23, 24). Thus, it is predicted that fluid secretion from serous cells in CF glands should be deficient to all mediators. However, because glands also contain mucous cells that do not appear to contain CFTR (12), the expected serous cell defect should be most easily detected if an agonist could be found that preferentially activated serous cell secretion.

Vasoactive intestinal peptide (VIP) stimulates macromolecular secretion from ferret submucosal glands by elevating [cAMP]i (25, 26) and degranulates ferret gland serous cells (26). In isolated submucosal glands from cats, VIP stimulates glycoconjugate release without stimulating contraction of myoepithelial cells (27). Binding sites for VIP are detected on human submucosal glands (28), and the Calu-3 human serous cell model has functional VPAC1 (VIP/PACAP-II) receptors (29). In
was 7.4, and osmolarity was adjusted to 290 mosM. A piece of ventral gland mucous secretion that reached a maximal value at about 1 min was used to control for the possibility that VIP receptors are decreased in CF tissues. After intervals of 40 min to 1 h, 10 μM carbachol was then added to test for gland viability. In the second procedure, we first stimulated transiently with a 2.5 μM solution of carbachol until small bubbles of mucus formed over some gland ducts and then repeatedly replaced the bath with fresh Krebs-Ringer bicarbonate buffer until secretion either stopped or returned to basal values. VIP + forskolin was then applied, and the secreted droplets of mucus were followed for at least 40 min to detect any slight increase in the rate of secretion. A second application of 10 μM carbachol was then applied.

**Optical Measurements**—Bubbles of mucus within the oil layer were visualized by oblique illumination, and digital images were captured with a CCD sensor mounted on a microscope (small field) or were obtained directly with the macro lens of a Nikon digital camera (large field). For macro images, each image contained an internal reference grid to compensate for any minor adjustments in magnification made during the experiment. Stored images were analyzed either by direct measurement or with Scion Image software (Scion Corp., Frederick, MD). Mucous volumes were determined from the size of the spherical bubbles; bubbles that were not spherical were omitted from secretion rate analyses. Details of these methods are given in Refs. 30 and 31.

**Statistics**—Data are means ± S.E., and Student’s t test for unpaired data was used to compare the means of different treatment groups unless otherwise indicated. The difference between the two means was considered to be significant when p < 0.05.

**RESULTS**

Pieces of human tracheal or bronchial epithelia were prepared so that spherical bubbles of uncontaminated mucus formed within an oil layer on the surface (Fig. 2). The bubbles of mucus, which remained attached to the gland ducts, were optically monitored, and their volumes were estimated by assuming that they were spheres (31). Glands sometimes began secreting at room temperature or started secreting as the bath was warmed (Fig. 2a). This basal secretion was variable, usually diminished or stopped within 30 min after the tissue reached 37 °C, and in general was less pronounced than in sheep and pigs (30, 31).

**Stimulation of Donor Tracheal Glands and Non-CF Bronchial Glands with VIP/Forskolin**—In many glands, including pig bronchial submucosal glands (30, 32), agents that elevate [cAMP], produce secretion. In the pancreas and in the Calu-3 serous cell model (33, 34), fluid secretion produced by the elevation of [cAMP], is mediated by HCO$_3^-$ secretion and is CFTR-dependent. Prior studies of [cAMP]-mediated, serous cell protein secretion emphasized pathways through β-adrenergic receptors (16), but isoprotrenal produces minimal mucous secretion in sheep (31), pigs (30, 32), and humans, in contrast with VIP (30) and forskolin (32), so these latter agents were used.

Treatment of control tissues with VIP or forskolin resulted in gland mucous secretion that reached a maximal value at about 10 min and was then sustained (Figs. 2b and 3). Secretion in response to VIP or forskolin was observed in each of 15 donor...
tracheas and in bronchial glands from each of 10 subjects with non-CF diseases (100%).

**Secretion Rates**—Secretion rates and profiles in response to VIP/forskolin were quantified for a subset of glands in donors and other diseases (Fig. 3). For a subset of 68 glands from donor trachea, secretion rates varied from 0 to a maximal rate of 4.50 nl/min/gland with a mean rate of 1.04 ± 0.23 nl/min/gland. In a subset of 48 glands from non-CF diseases, secretion rates ranged from 0 to 5.02 nl/min/gland with a mean rate of 1.14 ± 0.35 nl/min/gland. As with responses to carbachol in sheep (31) and pigs (30), we observed wide variations in secretion rates to VIP/forskolin among glands within subjects (Figs. 2 and 3).

**Stimulation of CF Tracheal and Bronchial Glands with VIP⁄Forskolin**—In marked contrast with the gland secretory responses seen in all non-CF subjects, submucosal glands from CF subjects were completely refractory to stimulation with VIP or forskolin or to combined treatment (Figs. 4 and 5). We observed no gland secretion to either agent, alone or in combination, in any of eight CF subjects followed for periods of 40–60 min (p < 0.001 for CF versus either control group, Chi square). All of the glands counted as refractory to forskolin were otherwise functional because they secreted in response to the [Ca²⁺]-elevating agonist carbachol (Figs. 4 and 5) (35). The percentage of carbachol-responsive glands that also responded to forskolin was 73% for donor trachea (605/827 glands), 89% for bronchi from diseases other than CF (148/166 glands), and 0% for CF (0/450 glands) (Fig. 6).

**Time-dependent Changes in Gland Responsiveness**—In our animal studies of carbachol-mediated secretion, we observed no diminution in responsiveness for tissues up to 24 h after harvest. In human donor tissues, responses to carbachol also appeared to be undiminished for at least 24 h after harvest, but responses to VIP/forskolin were less robust and declined in responsiveness after ~10 h (Fig. 7), although some control glands were observed responding to forskolin for up to 40 h.
after harvest. The mean ages of tissues at the time of experiments for donors, other diseases, and CF were 15 ± 11, 13 ± 8, and 11 ± 9 h, respectively. Thus, CF tissues were tested on average several hours earlier than controls, eliminating a time-dependent change in responsiveness as a basis for the absence of responses in the CF tissues.

**Serous Cells Are Abundant in CF Airway Glands**—One possible explanation for the complete lack of cAMP-mediated secretion from CF glands is that such secretion is proposed to originate from serous cells, and conversion of serous to mucous cells has been observed in chronic bronchitis (13, 36). However, such conversion has not been claimed for CF glands, and histological examination of glands from the CF and non-CF subjects we studied showed abundant serous cells in the CF glands (Fig. 8). As reported by others (37–39), simple inspection revealed CF glands to be much larger than glands in the non-CF groups. Although we have not yet quantified the extent of CF gland hypertrophy in our samples, the volume of serous cells in the CF airways we studied is clearly greater than in control tissues, further highlighting the remarkable absence of responsiveness to VIP or forskolin.

**DISCUSSION**

We found that glands from cystic fibrosis airways completely lack secretion stimulated by VIP or forskolin. Our experiments with intact, individually monitored airway glands are the first to demonstrate a defect in CF airway gland volume secretion, but they were preceded by highly informative experiments with primary cultures of serous-like cells derived from CF airway glands, which showed defects in stimulus-evoked short circuit current (18) and fluid secretion (19, 40). Together with localization of CFTR to gland serous cells (12), the present and previous studies establish serous cell dysfunction as a consistent feature of CF submucosal glands.

In our study, serous cell dysfunction was not caused by general debility, inactivity, or inflammation because every non-CF transplant patient responded to VIP/forskolin stimulation. Because forskolin was also ineffective, a deficiency in VIP receptors cannot explain the results, nor can serous-mucous cell conversion because direct inspection revealed abundant serous cells within CF submucosal glands.

The absence of [cAMP]$_i$-mediated serous cell fluid secretion in CF glands generates a series of questions about gland secretion, which are discussed with reference to the model of gland secretion shown in Fig. 9. In that model, gland mucus is the joint product of serous cells and mucous cells, which normally secrete together. In the following discussion, we distinguish between fluid secretion, which we use as shorthand for electrolyte-driven water transport, and macromolecular secretion, which refers to the secretion of everything else, including mucus and non-mucin proteins. Normal mucus is ~98% water.

**What Is the Mechanism of Serous Cell Fluid Secretion?**—Our results are consistent with the serous cell model developed by studies of Calu-3 cells (22) and primary cultures of serous cells...
airways is maximally stimulated by ACh, that forskolin produces about 60% of that volume, and that the two agonists are not additive. CFTR expression, studied with a well characterized polyclonal antibody and with in situ hybridization, was observed in serous but not mucous tubules (12), although others have reported more extensive expression (42). If CFTR is localized to serous cells, then the absence of forskolin-stimulated secretion in CF glands suggests that normal mucous cells do not secrete fluid in response to forskolin, whereas the continued ability of CF glands to secrete to ACh suggests that fluid secretion by mucous cells does not require CFTR.

How Is Macromolecular Secretion Linked to Fluid Secretion?—Most prior studies of secretion by cultured gland cells or explants have studied either the release of labeled macromolecules or short circuit current, but these two features of secretion may be uncoupled, especially in glands expressing a genetic defect. Prior studies with explants of whole bronchial segments (43) or primary submucosal gland cell cultures (44) from CF subjects found defective stimulus-evoked secretion of macromolecules. We have not addressed that possibility in the present experiments.

Is Secretion of CF Glands to Cholinergic Agents Normal?—Given the total loss of secretion to VIP and forskolin in CF glands, is the secretion that remains to carbachol indistinguishable from normal secretion? That is, can this Ca\(^{2+}\)-elevating agent induce CF serous cells to secrete? The model of gland function shown in Fig. 9 predicts that it cannot, resulting in gland fluid secretion to cholinergic agents that will be mediated only by fluid secretion from mucous cells. It is not a simple matter to determine whether such secretion is deficient. As documented by others, the CF glands in our study appeared much larger than normal, but we have not yet established that point with morphometry. Given the expected hypertrophy of CF submucosal glands (a 4-fold increase was observed in a recent study (39)), a meaningful comparison of secretion rates requires that the rates be expressed relative to gland volumes. Such studies are now underway.

Does the Composition of CF Gland Mucus Differ from Normal?—Surprisingly, secretions of pig glands in response to forskolin or carbachol had equivalent pH values (30), and again surprisingly, [Na\(^{+}\)] and pH values for carbachol-stimulated gland secretions were equivalent in CF and control subjects (35). Whether the loss of CFTR will alter protein secretion from serous cells in situ, as occurred for cultured CF gland cells (44), is uncertain. It remains possible that the only component missing from CF mucus is CFTR-dependent salt and water flux.

Does VIP Stimulation of Glands Play an Anti-inflammatory Role?—VIP is one of the most abundant peptides in the lungs, and a plethora of studies have implicated VIP pathways in the suppression of inflammation and cell damage (45). Exactly how this occurs is unknown. If selective VIP activation of serous cells occurs naturally, it is possible that it could contribute to the anti-inflammatory role of VIP in the airways, and its loss in CF may contribute to the heightened inflammatory state that is a hallmark of CF airway disease (46, 47).

Does the Gland Defect Help Explain the Initiation of Infections in CF Airways?—People with cystic fibrosis die primarily because their lungs become chronically infected with bacteria and fungi that are easily cleared from normal lungs. Importantly, the pathogens in CF airways are trapped within the mucus (8), but the ability of even normal mucus to inhibit the growth of pathogens is incomplete and wanes over time, emphasizing the importance of mucociliary clearance and cough in limiting the residence time of pathogens in the airways (48). The loss of serous cell secretion may dispose the airways to infections via multiple mechanisms, including slower transport

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**Fig. 8. Serous cells are abundant in CF submucosal glands.** A 5-μm paraffin section through formalin-fixed bronchial tissue stained with Hematoxylin and Eosin. a (inset), gland at lower magnification, with a portion of the duct opening onto the airway surface at top. Tissue had been stimulated with both forskolin and carbachol. The surface epithelium is missing, and the cartilage, which would normally have occupied the semicircular areas, was micro-dissected away to allow mounting of the flat epithelial sheet required for optimal optical measurements. b, higher magnification of the boxed area of the gland, showing serous (S) and mucous (M) tubules.

**Fig. 9. Schematic model of submucosal gland.** Four functional compartments have been proposed based on anatomical and immunohistochemical data with CFTR located primarily in serous cells. Pathways that elevate [Ca\(^{2+}\)], such as ACh, are hypothesized to activate fluid and macromolecular secretion from both serous and mucous cells. Pathways that elevate [cAMP], such as VIP, are hypothesized to stimulate serous cells and mucin but not fluid secretion from mucous cells. The CFTR-dependent fluid-secreting pathway is deleted in cystic fibrosis glands.

(18, 19), in which CFTR is required for anion secretion to all mediators (see Introduction). Secretion in response to agents that elevate [Ca\(^{2+}\)], is effected either because CFTR is normally open or because stimulation releases ATP and activates CFTR via an apical autocrine pathway (41).

**What Is the Mechanism of Mucous Cell Fluid Secretion?**—Ballard and colleagues (32) showed that mucus secretion by pig...
of mucus, absolute stasis of mucus caused by tethering to glands (49), reduced secretion of antimicrobials and anti-inflammatory agents, and reduced bioavailability of these agents because of inadequate dispersal (50). All of these features will be exacerbated by increased fluid absorption and decreased fluid secretion by the surface epithelium (6, 51).

Summary—Based on the above results and reasoning, our working hypothesis is that secretion by CF submucosal glands lacks the electrolyte-driven fluid component normally supplied by serous cells. Macromolecular secretion by both serous and mucous cells, as well as electrolyte-driven fluid secretion by mucous cells, are hypothesized to be intact. Because the rheologic properties of mucus depend critically on the concentration of macromolecules during initial formation of the gel and are resistant to subsequent changes, we hypothesize that a deficiency in electrolyte-driven water transport deep within the mucous cells, as well as electrolyte-driven fluid secretion by serous cells. Absent gland secretion to VIP in Cystic Fibrosis Airways

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