Submucosal gland dysfunction as a primary defect in cystic fibrosis

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ABSTRACT

It has been proposed that defective submucosal gland function in CF airways is a major determinant of CF airway disease. We tested the hypothesis that submucosal gland function is defective early in CF subjects with minimal clinical disease. Functional assays of gland fluid secretion rate and viscosity were performed on freshly obtained nasal biopsies from 6 CF subjects and 5 non-CF controls (age range 2–22 years). Secretions from individual submucosal glands were visualized by light/fluorescence microscopy after orienting and immobilizing biopsy specimens in a custom chamber. The viscosity of freshly secreted gland fluid after pilocarpine, measured by fluorescence recovery after photobleaching of microinjected FITC-dextran, was 4.9 ± 0.2- vs. 2.2 ± 0.2-fold greater than water viscosity in CF vs. non-CF specimens, respectively (SE, $P<10^{-4}$). Gland fluid secretion rate in CF specimens, measured by video imaging (4.5±0.5 nL/min/gland, $n=6$), was 2.7–fold reduced compared to non-CF specimens ($n=3$, $P<0.05$). Quantitative histology revealed similar size and morphology of submucosal glands in CF and non-CF specimens. Our results suggest that defective airway submucosal gland function is an early, primary defect in CF. Therapies directed at normalizing gland fluid secretion early in CF may thus reduce lung disease.

Key terms: CFTR • airway • mucus • fluorescence microscopy • photobleaching

Cystic fibrosis (CF) is the most prevalent lethal genetic disease in Caucasians. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a cAMP-regulated membrane Cl− channel in epithelia in the airways, pancreas, and other tissues. How defective CFTR function causes lung disease in CF remains a major unresolved issue with important consequences for new therapies. A variety of mechanisms have been postulated to relate defective CFTR to CF lung disease, including abnormalities in airway surface liquid (ASL) ionic composition, pH, oxygenation and volume, intrinsic hyperinflammation, and defective airway submucosal gland function (1–5). Airway submucosal glands secrete a mixture of salts, water, and macromolecules onto the airway surface, establishing ASL composition and physical properties and providing antimicrobials and mucins
for bacterial defense and airway lubrication (Fig. 1A). Watery fluid secreted by serous glandular acini passes through mucous tubules where mucins are added. The protein-rich fluid then passes through a collecting duct and onto the airway surface.

CFTR is expressed at the luminal surface of serous epithelia in airway submucosal glands (6, 7). Primary cell culture models have shown reduced chloride currents and fluid secretion in CF human serous gland cells (8–10). Application of CFTR inhibitors to normal airways reduces water fluid secretions from submucosal glands and increases the viscosity of secreted fluid (11, 12). The defective gland hypothesis postulates reduced secretion of watery fluid by submucosal glands in CF with unimpaired or increased protein secretion. The consequent hyperviscous glandular secretions in CF are postulated to produce lung disease by impairing mucociliary clearance and antimicrobial activity, leading to chronic infection, inflammation, and airway destruction. The principal evidence for defective gland function in CF is the finding of hyperviscous and reduced gland fluid secretions in CF airways obtained at the time of lung transplantation (13, 14). However, it has not been possible to determine whether defective gland function in CF is a primary or secondary consequence of defective CFTR function because CF airways obtained at the time of lung transplantation are severely damaged, and airway submucosal glands may be hypertrophied secondary to chronic infection, inflammation, and obstruction. Also, in some (but not all) studies, abnormal airway submucosal gland morphology with obstructive changes is found in young CF subjects before significant clinical disease (15–18), consistent with defective gland function as a primary abnormality in CF. We reasoned that demonstration of defective submucosal gland function very early in CF would provide the best evidence for defective gland function as a primary abnormality in CF.

We asked whether submucosal gland function is defective in early CF prior to significant clinical disease and secondary changes in gland physiology. Functional studies were performed on freshly obtained nasal airway biopsies from pediatric CF and non-CF subjects. Nasal rather than lower airway tissue was sampled to obtain adequate numbers of intact glands for functional measurements. Submucosal glands in the nasopharyngeal cavity have similar physiology to glands in lower airways and are in an environment with less exposure to infection and inflammation. Novel methods were developed to measure the physical properties of freshly secreted fluid from individual glands in the biopsy specimens. Remarkably increased viscosity and reduced fluid secretion was found in secreted fluid in CF vs. non-CF specimens, providing direct evidence for an intrinsic defect in submucosal gland function in CF. We propose that reduced submucosal gland fluid secretion is an important initiating and sustaining factor in CF lung disease, suggesting gland fluid secretion as a target for development of new CF therapies.

MATERIALS AND METHODS

Nasal airway biopsy

Nasal biopsies were performed by an experienced ear-nose-throat surgeon on pediatric subjects during previously scheduled endoscopic sinus surgery, and on control (non-CF) subjects undergoing adenotonsillectomy for airway obstruction. Blakesley thru-cut, straight, 13 cm sinonasal biopsy forceps were used to obtain ~4 mm fragments of mucosa and underlying tissue from the anterior-inferior turbinate bilaterally (Fig. 1B). Bleeding generally ceased spontaneously by completion of adenotonsillectomy, but in some cases suction cautery was used
to achieve complete hemostasis. No subject reported pain at the biopsy site, and no subsequent postoperative care was required. All biopsy sites were confirmed to be healed at a three-week postoperative follow-up visit. All procedures were approved by the U.C.S.F. Committee on Human Research.

**Tissue preparation for functional measurements**

Nasal biopsies were obtained in the operating room and immediately transported to the laboratory in ice-cold HCO$_3$-buffered Kreb’s solution (in mM: NaCl 120, NaHCO$_3$ 25, KH$_2$PO$_4$ 3.3, K$_2$HPO$_4$ 0.8, MgCl$_2$ 1.2, CaCl$_2$ 1.2, glucose 10, pH 7.4). A biopsy specimen was removed from the solution, and the smooth mucosal surface was identified using a stereo-dissection microscope and cleaned with a cotton swab. For embedding, 3 ml of agarose (3% in HCO$_3$-Kreb’s containing 50 µM pilocarpine) at 45°C was placed in a small Petri dish. After cooling to 39°C, the specimen was placed submucosal side down into the agarose and temperature was reduced to 37°C in a few seconds with an air stream, which solidified the agarose (Fig. 1C). The dry mucosal surface was covered with mineral oil (50 µl/cm$^2$ tissue) and transferred to a 37°C microincubator in 95% air/5% CO$_2$.

**Measurements of gland fluid secretion rate and viscosity**

Based on early work of Quinton (19), gland fluid droplets under oil were imaged by light microscopy with side-illumination using a Nikon SMZ1500 stereo-microscope. Serial images were acquired using a ×1.6 objective lens and digital camera (Pixel Link A642). Secretion rate was computed from increasing droplet diameter, assuming hemispheric droplet geometry as described previously (20). Gland fluid viscosity was measured by photobleaching of fluid droplets microinjected with 10 kDa FITC-dextran (2.3–4.6 nl of a 20 mg/ml solution) using a glass microneedle, which resulted in a <1% dilution of gland fluid. The output of an argon-ion laser (488 nm) was modulated by an acousto-optic modulator to apply a brief (<1 ms) bleach pulse of ~5000-fold greater intensity than the probe beam. The laser beam was directed by a dichroic mirror (510 nm) and focused onto the gland by a ×50 extra-long working distance air objective (numerical aperture 0.55, working distance 8.7 mm). Emitted fluorescence was filtered (515 nm long-pass), detected by a photomultiplier, amplified, and digitized by a 14-bit analog-to-digital converter. Recovery half-times ($t_{1/2}$) were determined from fluorescence recovery curves, F(t), using the equation: $F(t) = F_o+\left[F_o+R(F_{inf}-F_o)\right]/\left[1+(t/t_{1/2})\right]$, where $F_o$ is pre-bleach fluorescence, $F_{inf}$ is fluorescence at infinite time, and R is the fractional fluorescence recovery (21). Individual gland fluid droplets bleached in different locations (5–16 measurements per droplet) were averaged to generate single recovery curves.

**Histology**

Nasal biopsy samples were fixed for at least 2 h in buffered formalin (10%) and stored in 0.1 M phosphate buffer (pH 7.4). Standard tissue dehydration and paraffin infiltration was performed in a Tissue-Tek VIP processor (Sakura Finetek, Torrance, CA). Sections were cut at 4 µm on a rotary microtome, transferred to glass slides, and stained with hematoxylin and eosin.
Sections were viewed and photographed on an Olympus light microscope equipped with a digital imaging system (QImaging, Burnaby, Canada). Area measurements were performed on a Leica AS LMD microsystem (Leica Micosystems, Wetzlar, Germany).

RESULTS

Table 1 summarizes the clinical characteristics of normal and CF subjects from which nasal biopsies were obtained. The age range was 2–22 years. Three of the six CF subjects had very mild disease as evidenced from the forced expiratory volume at one second (FEV₁) of >75 percent predicted, Shwachman clinical score >75, and Brasfield radiological score >20. Nasal biopsies from five non-CF subjects were obtained at the time of tonsillectomy/adenoidectomy surgery.

Biopsy specimens were processed immediately after procurement for quantitative measurement of gland fluid viscosity and secretion rate. Specimens were oriented with mucosal surface upward and immobilized in an agarose gel (Fig. 1C). The mucosal surface was covered with mineral oil to visualize freshly secreted fluid droplets over gland orifices (Fig. 1D). The light micrograph at the bottom shows multiple fluid droplets at the mucosal surface of a biopsy specimen from a normal subject at 10 min after stimulation by pilocarpine.

The viscosity of gland fluid secretions was measured from the kinetics of fluorescence recovery after photobleaching of fluorescently stained gland fluid droplets. Fig. 2A (top) shows the principle of the approach. The fluorescence in a cylindrical region of a fluid droplet was irreversibly bleached by a briefly applied intense laser beam. The subsequent increase in fluorescence results from diffusion of unbleached FITC-dextran into the darkened region, providing a quantitative measure of FITC-dextran diffusion coefficient and hence fluid viscosity. Viscosity was computed from the ratio of the recovery rates in saline (Fig. 2A, bottom, right) vs. in gland fluid. The half-time (t₁/₂) for fluorescence recovery of FITC-dextran in saline was 35±1 ms.

Individual gland fluid droplets were microinjected with FITC-dextran for photobleaching measurements as shown in the fluorescence micrograph in Fig. 2A (bottom, left). It was noted during the microinjections that droplets in all CF biopsy specimens were remarkably more adherent to the glass micropipette than those in specimens from normal subjects. Fig. 2B shows representative fluorescence recovery curves for fluorescently stained gland fluid droplets in nasal biopsies from three normal and three CF subjects. Fluorescence was bleached to ~70% of initial fluorescence intensity by application of a brief (<1 ms) intense laser beam. Fluorescence recovery in gland fluid from normal subjects was relatively rapid (t₁/₂=77±5 ms, SE, n=4 subjects) and essentially complete, as seen by the similar fluorescence prior to and at long times (2–12 sec) after application of the bleach pulse. Fluorescence recovery was significantly slowed in gland fluid from CF subjects with t₁/₂ of 172 ± 7 ms (n=6 subjects). Figure 2C shows averaged t₁/₂ and corresponding relative fluid viscosities for each normal and CF subject (labeled as in Table 1), together with averaged results. There was no relation of t₁/₂ to age or severity of illness. Gland fluid in CF nasal biopsies was ~2.2 times more viscous than that from biopsies from normal subjects.
Secretion rates for individual gland fluid droplets were measured from the kinetics of droplet growth (assuming hemispherical shape) as observed by light microscopy. Fig. 3A (inset) shows serial micrographs of gland fluid droplets from a normal and CF biopsy specimen. Fig. 3A shows the kinetics of droplet expansion of all measured fluid droplets averaged for each subject. All fluid droplets in sharp focus in the images were included in the analysis. Fig. 3B gives corresponding secretion rates determined from linear regression of droplet growth data. Gland fluid secretion rate was significantly reduced ~2.7-fold in CF compared to normal nasal biopsies.

Gland histology in biopsy specimens was assessed by light microscopy of hematoxylin and eosin-stained paraffin sections. Fig. 4A (top) shows low magnification of a biopsy with the mucosal surface and a submucosal gland indicated. Higher magnification of glands of normal (Fig. 4A, bottom, left) and CF (right) specimens showed similar morphology. As examined by a lung pathologist blinded to genotype information, no abnormalities were seen except for a biopsy from one CF subject (CF2) where dilatation of gland acini was seen. Fig. 4B shows that the fractional gland area in biopsy specimens was not significantly different between the control and CF groups.

**DISCUSSION**

We found that fluid secretion rate from submucosal glands was reduced 2.7-fold and secreted fluid viscosity was elevated 2.2-fold in early CF, prior to significant clinical disease and glandular pathology in most of the subjects. In addition, there was no relation of age or severity of illness to gland secretion rate or viscosity in the CF subjects. Further, the results here are similar to those reported previously in airways from older, more severely affected CF subjects (13). These results provide evidence for submucosal gland dysfunction as an intrinsic defect in CF, extending previous findings supporting the involvement of CFTR in gland fluid secretion in intact airways (11, 12, 22), and of reduced fluid secretion and hyperviscosity in severely diseased CF airways (13, 14). Glandular dysfunction may thus be a primary factor in the initiation and progression of CF lung disease, perhaps in combination with other abnormalities that have been proposed to exist in CF, including accelerated airway surface liquid absorption due to ENaC hyperfunctioning (23, 24) and/or airway hyperinflammation (25). Absence of functional CFTR in CF causes a reduction in the fluid component of gland serous secretions, resulting in increased viscosity of the fluid secreted onto the airway surface with consequent impaired bacterial clearance by mucociliary mechanisms (1, 4, 5, 26).

Our experimental strategy was to use optical methods to measure the properties of freshly secreted fluid from submucosal glands in nasal biopsies. Fluid droplets were visualized during their appearance and growth from gland openings under oil, without modification by airway surface cells or contamination by airway surface fluid. Based on prior studies (13, 14), pilocarpine was used as the agonist to stimulate gland fluid secretion, as little fluid is secreted by CF glands in response to cAMP agonists. In those prior studies, it was not possible to establish or quantify the impairment of fluid secretion in CF glands because of marked glandular hypertrophy and thus uncertainty in functional glandular epithelial surface area. The 2.7-fold reduced rate of pilocarpine-induced fluid secretion from glands in the CF nasal specimens suggests that most but not all fluid secretion is CFTR-dependent. Indeed, nasal secretions can be induced in CF subjects after chewing chili peppers (27), although there are likely multiple sources and mechanisms of chili pepper-induced nasal secretions.
A major challenge in these studies was the development of methods to measure gland fluid secretion and properties in small nasal biopsy specimens. Biopsy specimens were oriented and immobilized in agarose to access the mucosal surface for visualization of fluid secretion. Generally, the nasal biopsy specimens contained eight or more intact, fluid-secreting submucosal glands. All measurements on biopsies were completed within 1–2 h. In control studies, tissues were found to remain viable after immobilization in agarose for at least 6 h as demonstrated by the ability to induce fluid secretions repeatedly after washing the mucosal surface. In preliminary studies it was generally not possible to identify intact, secreting glands in endobronchial biopsies from the lower airways of pediatric subjects obtained during flexible bronchoscopy. The lower airway biopsy specimens were small and distorted by the sampling process, in contrast to the specimens obtained by nasal biopsy, which contained many intact submucosal glands and provided a reasonably flat surface for optical measurements.

Our results focus attention on submucosal glands as a target for drug therapy in CF. In addition to approaches that replace or correct mutant CFTR in all tissues, such as gene or small-molecule therapies, approaches that target submucosal glands specifically may be useful, such as activation of non-CFTR chloride or other channels involved in gland fluid secretion or inhibition of mucus production by glands. Correction of the glandular phenotype in CF—reduced fluid secretion and hyperviscosity—may thus reduce the pulmonary manifestations of the disease.

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REFERENCES


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**Table 1**

Clinical characteristics of study subjects

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<th>CF Subjects</th>
<th>Age, gender</th>
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CF, cystic fibrosis

<table>
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<td>Chronic cough</td>
<td>T &amp; A and BAL</td>
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N, normal; OSA, obstructive sleep apnea; T & A, tonsillectomy and adenoidectomy; BAL, bronchoalveolar lavage
Figure 1. Quantitative microscopy measurements on nasal biopsy specimens. A) Schematic of airway submucosal gland showing watery fluid secreted by CFTR-expressing serous acini, addition of glycoproteins by mucous tubules, and passage of fluid onto the airway surface through a collecting duct. B) Location of biopsied region in anterior-inferior nasal turbinate. T, tongue; P, palate; MT, middle turbinate. C) Schematic showing nasal biopsy specimen immobilized in an agarose gel with mucosal surface facing upward. D) (Top) Photograph showing oil-covered biopsy specimen. (bottom) Micrograph of freshly secreted gland fluid droplets visualized under oil by brightfield microscopy and side-light illumination.
Figure 2. Increased gland fluid viscosity in CF as measured by fluorescence recovery after photobleaching. A) (top) Schematic showing cylindrical bleached region in a fluorescently stained gland fluid droplet. (bottom, right) Fluorescence recovery after bleaching 10 kDa FITC-dextran in saline showing rapid reduction in fluorescence after laser bleaching, followed by increasing fluorescence (recovery) as unbleached dye diffuses into the bleached region. (bottom, left) Fluorescence micrograph of FITC-dextran stained gland fluid droplets. B) Representative fluorescence recovery data for 10 kDa FITC-dextran stained gland fluid droplets from three normal (left) and three CF (right) subjects (6–8 recovery curves averaged per subject). Note the slower recovery in CF. C) Recovery half-times ($t_{1/2}$, left axis) fitted as described in Materials and Methods, and corresponding fluid viscosities (right axis), shown for biopsies from indicated normal and CF subjects (mean ± SE, 3–6 glands per subject, solid symbols), along with average for all subjects (± SE, open symbols). * $P < 10^{-4}$. 
Figure 3. Reduced gland fluid secretion rate in CF. A) Kinetics of droplet volume increase averaged from individual fluid droplets from normal and CF subjects. (Inset) Brightfield microscopy showing expansion of fluid droplets in biopsy specimen from normal and CF subject. Zero time corresponds to removal of mucosal surface fluid and covering the surface with oil. B) Averaged secretion rates (mean ± SE, 2–4 glands per subject, solid symbols), along with average for all subjects (± SE, open symbols). * P < 0.05.
Figure 4. Histology of submucosal glands in nasal biopsies. A) (top) Low magnification of gland biopsy from a normal subject showing epithelial mucosal surface (black arrows) and submucosal gland (white arrow). (bottom) High magnification of glands from normal (left) and CF (right) biopsy. Arrows indicate mucous tubules. B) Fraction of total volume occupied by submucosal glands determined by quantitative light microscopy (± SE, n=5 normal and 6 CF subjects). Difference not significant.