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Evidence for Periciliary Liquid Layer Depletion, Not Abnormal Ion Composition, in the Pathogenesis of Cystic Fibrosis Airways Disease

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Summary

The pathogenesis of cystic fibrosis (CF) airways infection is unknown. Two hypotheses, "hypotonic [low salt]/defensin" and "isotonic volume transport/mucus clearance," attempt to link defects in cystic fibrosis transmembrane conductance regulator–mediated ion transport to CF airways disease. We tested these hypotheses with planar and cylindrical culture models and found no evidence that the liquids lining airway surfaces were hypotonic or that salt concentrations differed between CF and normal cultures. In contrast, CF airway epithelia exhibited abnormally high rates of airway surface liquid absorption, which depleted the periciliary liquid layer and abolished mucus transport. The failure to clear thickened mucus from airway surfaces likely initiates CF airways infection. These data indicate that therapy for CF lung disease should not be directed at modulation of ionic composition, but rather at restoring volume (salt and water) on airway surfaces.

Introduction

Despite the advances in the genetics and molecular basis of cystic fibrosis (CF) (Davis et al., 1996), there is no clear understanding of the pathogenesis of CF lung disease. The phenotype is expressed in the airways of the CF lung and is reflected in unrelenting bacterial infections that account for >95% of the deaths from this syndrome (Davis et al., 1996). Two conceptual frameworks have been developed that describe the pathogenesis of CF lung disease. One focuses on the cell biologic effects of mutant cystic fibrosis transmembrane conductance regulator (CFTR), leading to speculations of increased binding of bacteria to CF airway epithelial cells (Imundo et al., 1995), failure of CF airway epithelial cells to ingest and kill luminal bacteria (Pier et al., 1996), and/or dysregulation of the CF airway inflammatory response (Konstan and Berger, 1997). The second, focusing on the known ion transport functions of CFTR, has attempted to link ion transport defects to the initiation of CF lung disease (Quinton, 1990; Boucher, 1994a).

Although the ion transport framework may be more parsimonious and consistent with the pathogenesis of other organ-level phenotypes of CF (Quinton, 1990; Davis et al., 1996), there is surprisingly no accepted description of the interrelationships between epithelial ion transport, airway surface liquid (ASL) physiology, and airways defense in the normal lung. At present, two theories attempt to link airway epithelial ion transport to lung defense and predict how mutations in CFTR adversely affect these relationships. One theory, the "hypotonic (low salt) ASL/defensin" hypothesis, postulates that normal airway epithelia are covered by an ASL with a [NaCl] sufficiently low (≤ 50 mM NaCl) to activate defensins and create an antimicrobial "shield" on airway surfaces (Quinton, 1994; Smith et al., 1996; Goldman et al., 1997). Generation of a low [NaCl], hypotonic ASL reflects selective transepithelial absorption of salt but not water from ASL, presumably a consequence of putative airway epithelial water impermeability or surface forces (Quinton, 1994; Widdicombe, 1997; Zabner et al., 1998). Defensin inactivation by iso- or hypertonic ASL (i.e., [NaCl] > 100 mM) in CF is postulated to reflect the Cl^- impermeability of CF epithelial cells (Smith et al., 1996; Goldman et al., 1997). The second theory, the "isotonic volume transport/mucus clearance hypothesis," predicts that airway epithelia regulate the volume (height) of ASL by isotonic ion and water transport to optimize mucus clearance (Boucher, 1994a). In CF, the rate of isotonic ion and water (volume) transport is abnormally high, which is predicted to reduce ASL volume, concentrate mucus, and lead to abnormal mucus transport and retention of tenacious mucus masses (plaques) in airways that serve as the nidus of infection (Jiang et al., 1993; Boucher, 1994b). It has been difficult to differentiate between these two theories of airway physiology because experimental models did not simulate the integrated physiology of the very shallow (~30 μm thick) ASL compartment.

We developed two human airway epithelial cell culture models to help distinguish between the two theories. The hollow fiber ("biofiber") culture system recreates the cylindrical geometry of airways and is well suited for studies of epithelial ASL volume transport/compositional regulation (Grubb et al., 1997). Our air–liquid interface planar preparation offers the unique feature of in vitro rotational mucus transport (Matsui et al., 1998). This model, coupled with microsampling/microelectrode techniques and confocal microscopy to measure the heights of both ASL compartments (i.e., the mucus layer, periciliary layer), allows us to study interrelationships among ASL ionic composition, ASL volume, and mucus transport. By measuring ASL ion composition, we tested key predictions of the hypotonic (low salt) ASL/defensin hypothesis: (1) that normal airway epithelia generate and maintain hypotonic (or low salt) solutions on airway surfaces, and (2) that CF airway surface liquid is isotonic (Joris et al., 1993) or hypertonic (with excess salt) (Gilljam et al., 1989). A second series of experiments tested key aspects of the isotonic hypothesis: (1) that normal airway epithelia are isotonic volume-absorbing epithelia that produce an isotonic ASL; (2) that CF airway epithelia absorb volume isotonically at abnormally high rates; and (3) that mucus transport is

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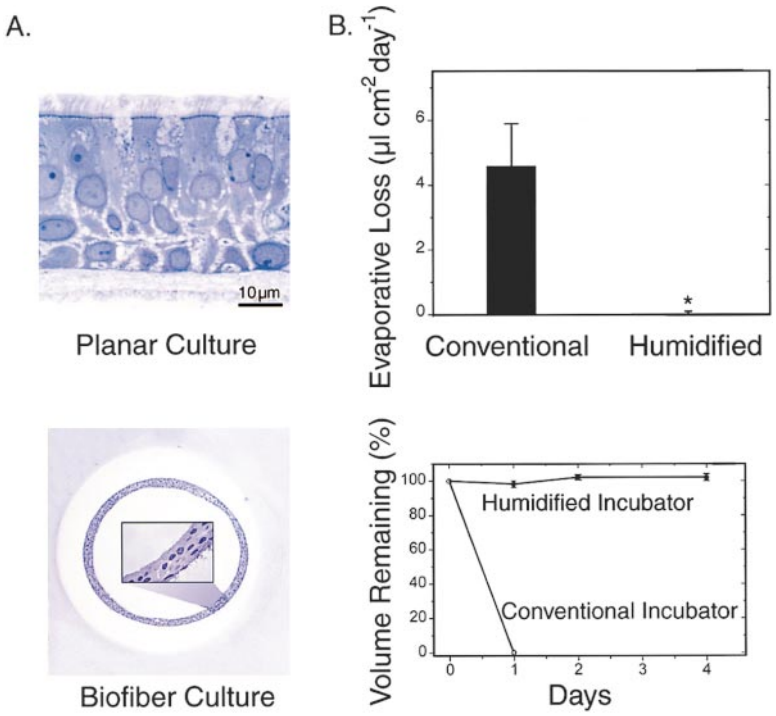

Figure 1. Human Airway Epithelial Cultures and Humidified Culture Chambers

(A) Human airway epithelial cell cultures. Top: Planar cultures exhibited a highly differentiated mucociliary phenotype, with ciliated cells interspersed with goblet cells. Bottom: The cylindrical structure of the biofiber culture and the multicellular epithelium in which a proportion of luminal cells were ciliated (inset).

(B) Chamber humidification. Top: Evaporative water loss from thin films of liquid as measured by the Oregon Green–dextran technique (* denotes $p < 0.05$). Bottom: Fate of 500 nl droplets of water maintained under humidified or standard incubator conditions.

deranged by depletion of the ASL through excessive isotonic volume absorption.

Results

Cell Culture Preparation and Humidification of Cell Culture Incubator

The planar air–liquid interface (ALI) cultures recapitulated the morphology of the well-differentiated epithelia that line normal airway surfaces (Figure 1A, upper panel). The morphology and transepithelial electrical resistances (R_t) of the CF planar cultures were not different from normals. The epithelial cells lining the biofibers (BF) were columnar, as in the ALI planar system, but were not as uniformly ciliated (Figure 1A, lower panel).

Conventional tissue culture incubators are not perfectly humidified. Because this interferes with the ability to make accurate compositional measurements of thin films of ASL over time, we developed a system (Experimental Procedures) to eliminate evaporative water loss. Data derived from impermeant fluorescent marker and 500 nl droplet techniques demonstrated that virtually no liquid is lost in the modified compared to a conventional incubator (Figure 1B).

Is Normal Airway Epithelial ASL under Basal Conditions Hypotonic (Low Salt) or Isotonic?

Direct measurements of ASL osmolality from normal airway epithelial planar cultures sampled at 24 hr (Figure $2A_i$), and BFs at 5 hr (Figure $2A_{ii}$), after test solution addition revealed that the liquid was isosmotic (referred to hereafter as "isotonic"). The chemical measurement of ASL Na^+ and Cl^- revealed values that exceeded 100 mM. In planar preparations, Cl^- concentrations were independently measured over the same interval with Cl^--selective microelectrodes and were also consistent with an isotonic ASL (pre: 137 ± 9.2 mM; post 24 hr: 128 ± 4.2 mM; n = 4). Cl^- measurements over an additional 24 hr revealed no significant changes (48 hr: 149 ± 17.4 mM; n = 4).

Can Normal Airway Epithelia Maintain Imposed Hypotonic Gradients?

Next, we asked if airway epithelia can maintain transepithelial hypotonic gradients when they are imposed across these epithelia. Two approaches were used.

In the first, 200 μl of either isotonic PBS (300 mOsm) or hypotonic PBS (1/3 normal PBS, 100 mOsm) containing the volume marker blue dextran (BD, 2%) was added to the surface of planar preparations (24 mm T-Cols) and the luminal liquid sampled with capillary pipettes over 24 hr. The osmolality of the added hypotonic solution rapidly became isotonic (Figure 3A). The equilibration to isotonicity reflected transepithelial osmotic water absorption as indicated by the increased volume absorbed from the lumen after the addition of hypotonic versus isotonic solutions (lower panel).

The second approach explored the rapidity of this response (Figure 3B). Twenty microliters of PBS or hypotonic PBS (100 mOsm) containing Oregon Green–dextran was added to either normal airway epithelial cultures (12 mm T-Cols) or MDCK cells (a water-impermeable renal epithelial cell line [Davis et al., 1998]) and ASL height and fluorescence intensity measured 10 min later by x-z confocal microscopy. Compared to isotonic PBS additions, ASL height of airway epithelia exposed to hypotonic PBS shrank by approximately two-thirds, consistent with a rapid equilibration to isotonicity (Figure

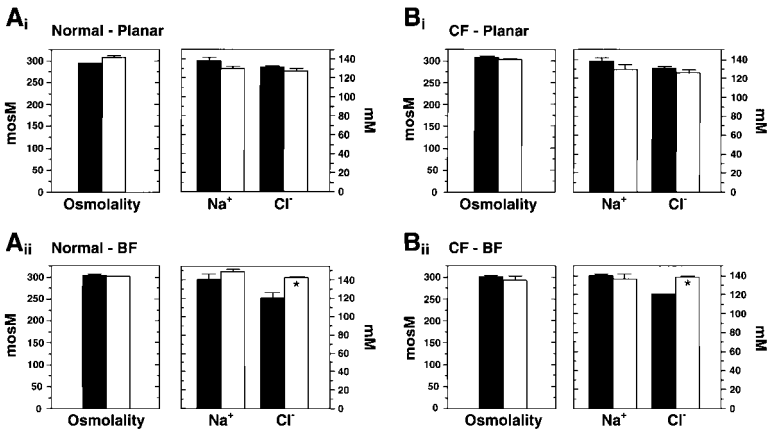

Figure 2. Composition of Airway Surface Liquid from Normal and CF Human Airway Epithelial Cultures

ASL was analyzed for both osmolality and ionic composition.

(A) Normal Cultures. (i) Planar. t = 0 (black bars) and 24 hr (white bars) after PBS addition (n = 8, osmolality; n = 16, ionic composition). (ii) Biofibers. t = 0 (black bars) and 5 hr (white bars) after PBS addition (n = 10). Comparable data for BFs were also obtained after 24 hr (Na^+ = 132 ± 2.7 mM; Cl^- = 132 ± 2.8 mM, n = 5).

(B) CF Cultures. (i) Planar. t = 0 (black bars) and 24 hr (white bars) after PBS addition (osmolality, n = 8; ionic composition, n = 6). (ii) Biofibers. t = 0 (black bars) and 5 hr (white bars) after PBS addition (osmolality, n = 3; ionic composition, n = 5). Note in both normal and CF biofibers bathed in KBR, [Cl^-] was slightly but significantly increased at 5 hr ($p < 0.01$).

3B, upper panel). This rapid equilibration reflected transepithelial osmotic water absorption from the ASL into the cell (note increase in cell height) and the basolateral bath (note increase in height and decrease in fluorescence) (Figure 3B). No acute changes in MDCK cell or basolateral bath height were detected after addition of hypotonic PBS (Figure 3B). Thus, both sets of experiments (Figures 3A and 3B) demonstrated that normal airway epithelia cannot maintain hypotonic solutions on their surfaces for prolonged periods.

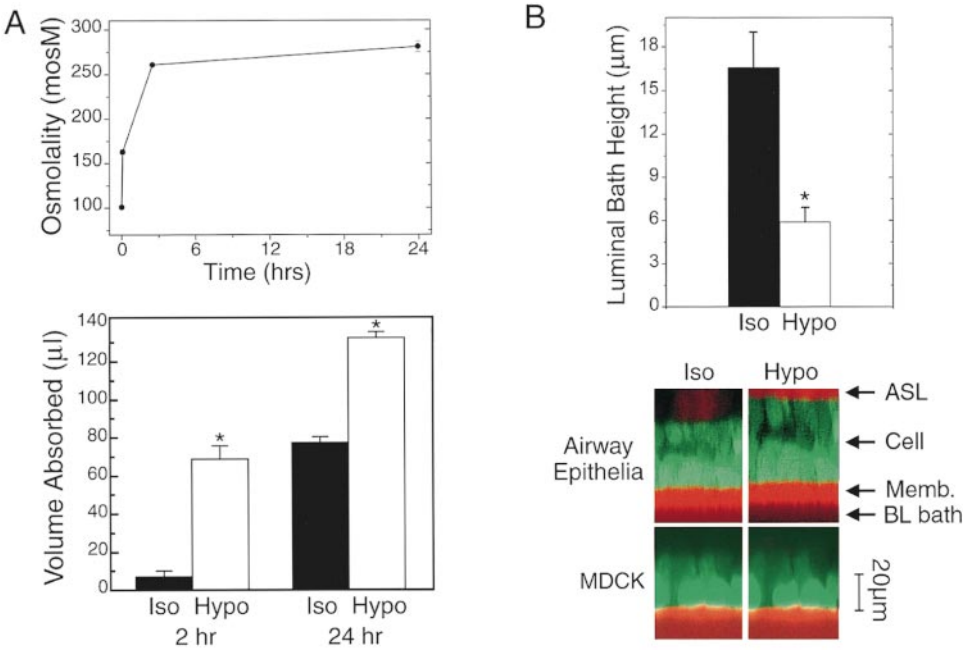

Figure 3. Response of Airway Epithelia to Hypotonic ASL

(A) Response to luminal hypotonic ASL challenge measured by microsampling. Hypotonic PBS (100 mOsm) was added to the luminal surface; 5 µl samples were obtained serially and analyzed for osmolality (upper panel) and volume absorbed measured by BD absorbance (lower panel). * denotes $p < 0.05$ isotonic versus hypotonic; n = 6.

(B) Optical measurements of transepithelial and cellular responses to hypotonic liquids. Upper panel: Summary data describing luminal surface liquid height 10 min after addition of hypo- or isotonic PBS to airway epithelia. N = 6; * denotes $p < 0.05$ hypotonic versus isotonic. In MDCK cell cultures, changes in luminal liquid height were undetectable after addition of either solution. Lower panels: x-z vertical images of planar human airway epithelial and MDCK cell cultures 10 min after 20 µl of hypotonic PBS (Hypo; 100 mOsm) or isotonic PBS (Iso; 300 mOsm) was added to the "lumen." In airway cultures, note that cell swelling (increase in height) occurred following hypotonic addition, TR-dextran fluorescence increased in the luminal compartment and decreased in the serosal compartment, all indicating a flow of water to the serosal compartment. No responses were detected in MDCK cells or in airway epithelia following isotonic additions.

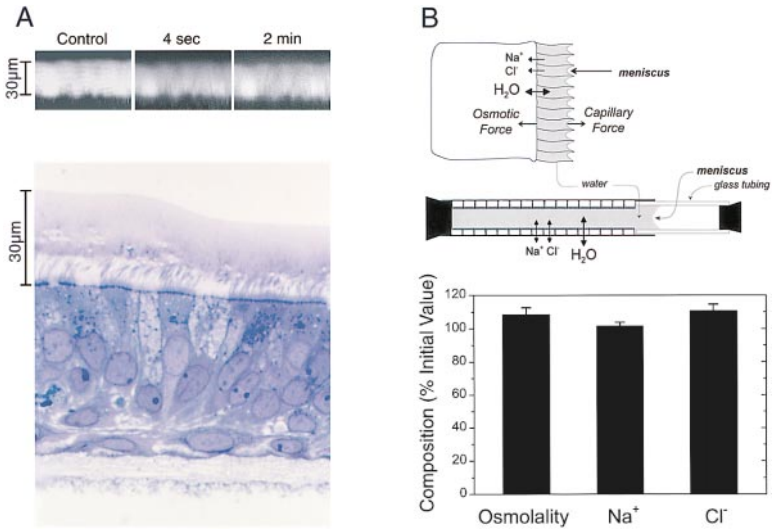

Figure 4. Studies of ASL "Surface Forces"

(A) Visualization of ASL surface topography. Upper panel: Fixation of ASL with perfluorocarbon/osmium (PFC/OsO_4). Confocal images of airway epithelial culture ASL labeled with Texas Red–dextran before (Control) and during fixation (4 sec and 2 min). Lower panel: Histology of an airway epithelial culture fixed by PFC/OsO_4. Note the mucus blanket in continuous contact with cilia tips.

(B) Functional model of capillary and hydrostatic forces. Upper panel: Diagram depicting postulated meniscus-associated hypotonic liquid production (Widdicombe, 1997). Top: Small radius (~0.1 μm) menisci formed between the tips of upright cilia generate capillary forces that offset osmotically driven water absorption from airway surfaces. Thus, NaCl but not water can be extracted from ASL. The "stopped flow" BF protocol (below cell model) had an air bubble at one end of the plugged system that forced absorption against a closed atmospheric system to simulate capillary–meniscus pressures. Lower panel: Osmotic and ionic composition of biofiber airway epithelial culture luminal contents in "stopped-flow" configuration. Luminal contents were sampled at time 0 and after 5 hr and data presented as percentage of the initial values: n = 4 (osmolality) or 3 (ionic composition).

Can Surface Capillary Forces Affect Airway Surface Liquid Tonicity?

As an alternative to absorption of salt across a water-impermeable epithelium to produce hypotonic ASL, it has been proposed that "surface forces" develop when ASL exists as a thin film on the surface of airway epithelia, as occurs in vivo, and retain water but not salt in ASL (Zabner et al., 1998). One such model postulated the formation of shallow menisci between the tips of outstretched cilia (Widdicombe, 1997). We tested this hypothesis in two ways.

First, we sought to morphologically identify menisci on planar culture preparations. Recent data suggest that osmium (OsO_4), which fixes cilia sufficiently rapidly to visualize metachronal waves (Satir, 1990), delivered in a hydrophobic vehicle (perfluorocarbon [PFC]) preserves ASL topography (Sims and Horne, 1997). To test the validity of this approach to search for menisci, we visualized PFC/OsO_4 fixation of the ASL compartment marked with fluorescent probes online with x-z confocal microscopy. ASL height was not perturbed by this fixation method (Figure 4A). Histological sections of this preparation revealed a mucus layer above a distinct periciliary liquid (PCL) compartment (Figure 4A, lower panel). With mucus covering the cilia, there is no opportunity for air/liquid menisci to form between the tips of "outstretched" cilia (Widdicombe, 1997).

Second, we designed a functional test of the hypothesis that capillary forces hold water but not salt on epithelial surfaces (Figure 4B). BFs under free-flowing conditions (with one unplugged end) absorb liquid isotonically (see Figure 5). With both ends of the BF plugged, the "stop-flow condition," the effect of meniscus formation is simulated by volume absorption against the obstructing plugs. Under "stop-flow" conditions, no volume absorption occurred, and the luminal liquid remained isotonic at both 5 hr (Figure 4B, lower panel) and 24 hr (Na^+ = 131 ± 2.3; Cl^- = 118 ± 1.7 mM, n = 7).

Is CF ASL Hypo-, Iso-, or Hypertonic?

We sampled ASL from CF planar preparations (Figure $2B_i$) and BFs (Figure $2B_{ii}$). The ASL on CF cultures was not hypertonic but isotonic with a [NaCl] similar to normals. These findings contrast with those of Gilljam et al. (1989) but are consistent with an absence of any known physiologic mechanism to generate hypertonic ASL (other than evaporation).

Do Normal Airway Epithelia Absorb Volume, and Is It Na^+ Dependent?

We tested for volume absorption with two techniques, one that bathed the lumen with an "unlimited" volume of liquid (BFs) and another that bathed the lumen with a "thin film" (planar cultures). Normal airway epithelial cells grown in BF cultures absorbed volume, and this transport was abolished by replacement of Na^+ (with NMDG) in the luminal solution (Figure 5). Normal airway

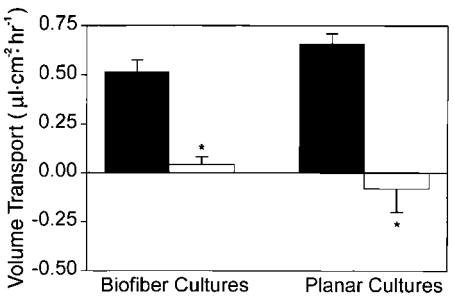

Figure 5. Absorption of Luminal Liquid by Normal Human Airway Epithelial Cells Grown in Biofibers or Planar Cultures

Volume transport by BFs (over 5 hr) and planar cultures (over 24 hr) was measured under control conditions (solid bars) and following replacement of luminal Na^+ by NMDG (open bars). Positive values indicate absorption, negative values secretion. BF control cultures, n = 15; Na^+ free, n = 7; planar control cultures, n = 20; Na^+ free, n = 6. *$p < 0.05$ control versus Na^+ free.

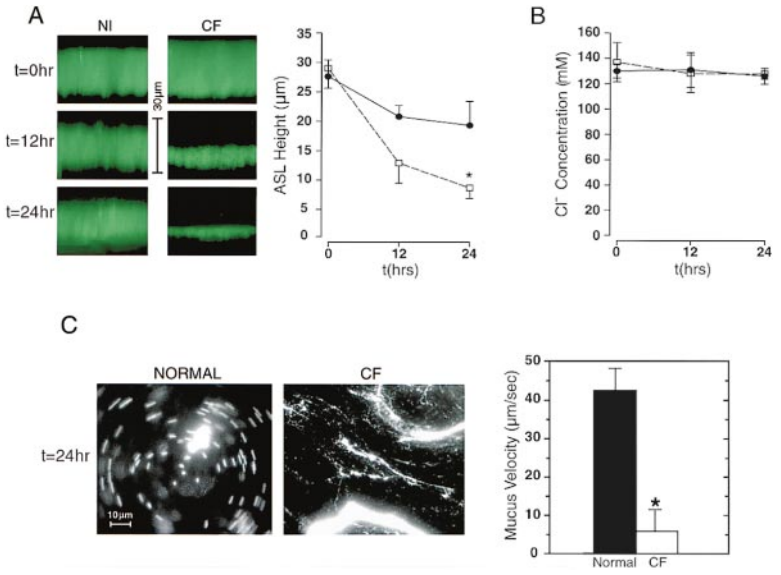

Figure 6. Comparison of ASL Height, Cl^- Concentration, and Rotational Mucus Transport in Normal versus CF Airway Epithelial Cultures

(A) ASL height measured immediately, 12 hr, and 24 hr after deposition of PBS containing Texas Red–dextran (pseudocolored green). Left: representative confocal microscopy images. Right: mean data for normal (circles) and CF (squares) ASL heights. Asterisk denotes CF ASL is significantly ($p < 0.05$; n = six/group) shallower than normal.

(B) Cl^- concentrations of normal (circles) and CF (squares) ASL measured with Cl^--selective microelectrodes over 24 hr (n = six/group).

(C) Long exposure (5 s) fluorescent micrographs of mucus (bead) rotational velocity 24 hr after administration of PBS/fluorescent markers. The normal culture exhibited arcs oriented around a central point consistent with rotation of beads in mucus; the CF culture demonstrated clumps of beads that did not move. The mean velocities for the normal and CF cultures at 24 hr are shown in the right panel (*$p = < 0.05$, CF different from normal; n = six/group).

epithelial cell planar cultures also absorbed volume from a thin film of ASL (~30 μm depth—see Figure 6), which also was sensitive to luminal Na^+ replacement (Figure 5). Analyses of ASL composition of BFs and planar cultures indicated that ASL volume transport was isotonic (see Figure 2 and below).

ASL Height, Cl^- Concentrations, and Mucus Transport in CF versus Normal Cultures under "Thin Film" Conditions

In a first series of experiments, ASL height, Cl^- concentration, and mucus transport were measured in CF and normal planar cultures for 24 hr after addition of fluorescent probes in PBS. CF cultures reduced ASL height at a significantly faster rate than normal cultures, consistent with accelerated volume absorption (Figure 6A). ASL Cl^- concentrations on CF or normal cultures did not change over time, indicating that volume absorption was isotonic (Figure 6B). Representative digital images of a normal culture at 24 hr acquired with a 5 s exposure demonstrated rotational mucus transport as lines (streaks) of fluorescence, whereas in the CF culture, mucus transport (38.3 ± 15 μm sec^{-1} at 12 hr) became stationary at 24 hr with mucus accumulating in plaque-like masses (Figure 6C—see graph on right for mean comparisons). Mucus transport in CF cultures at 24 hr was restored by addition of PBS (40 μl): 0 ± 0 μm sec^{-1} at 24 hr; 66 ± 23 μm sec^{-1} 10 min after PBS addition (n = 3).

To identify the mode of isotonic volume hyperabsorption by CF epithelia, the apical surfaces of CF cultures were exposed to 50 μl of either PBS or Na^+-free buffer (n = 6 each). Na^+-free buffer inhibited absorption (ASL height = 23.4 ± 4.0 μm pre; 19.7 ± 2.6 post 8 hr; n = 4) compared to control (ASL height = 25.4 ± 2.9 μm pre; 12.2 ± 2.1 μm post 8 hr; n = 4). Thus, like normal airway epithelia (Figure 5), most volume absorption by CF epithelia was Na^+ dependent.

How Does Increased Volume Absorption Lead to Reduced Mucociliary Clearance?

A second series of studies focused on the relationships among isotonic volume transport rates, mucus transport, and the heights (volume) of the individual ASL compartments (mucus, PCL). As in the initial studies, mucus transport persisted for 24 hr after addition of 50 μl of PBS containing tracers in normal but not CF cultures (mucus velocity = 26 ± 5 μm/s for normals [n = 4]; 1.2 ± 0.2 μm/s for CF [n = 4]; $p < 0.05$), and CF cultures exhibited excessive ASL volume absorption, as indicated by the shallower ASL height at 24 hr in CF (6.0 ± 1.0 μm, n = 4) compared to normal cultures (18.0 ± 2.6 μm, n = 4, $p < 0.05$). ASL height at 24 hr in CF cultures approximated that of outstretched cilia, that is, the PCL height (~6–7 μm), predicting that depletion of the PCL, with invasion of this compartment by mucus, interferes with mucus transport. Representative x-z images at 24 hr demonstrated beads within the 7 μm domain of the PCL in CF but not normal cultures (Figure 7A).

To further test this notion, we used the confocal microscope to localize mucin (with 1 μm beads) and cilia (with fluorescein-labeled lectins). At 24 hr after luminal volume addition, the beads resided atop the cilia in normal cultures, whereas in the CF cultures, beads colocalized with the cilia (Figure 7B).

Finally, to study in detail the interaction between cilia and PCL as a function of rates of volume absorption, we used the PFC/OsO_4 fixation technique to fix these normal and CF cultures 24 hr after luminal volume addition. In the normal cultures, which maintained mucus transport, electron micrographs (EMs) revealed an electron-lucent zone surrounding cilia (PCL), with cilia fully extended to interact with the inner surface of the overlying mucus blanket (Figure 7C). In contrast, in CF cultures at 24 hr when mucus transport was stopped, EMs revealed a shallow PCL layer. Within this depleted PCL

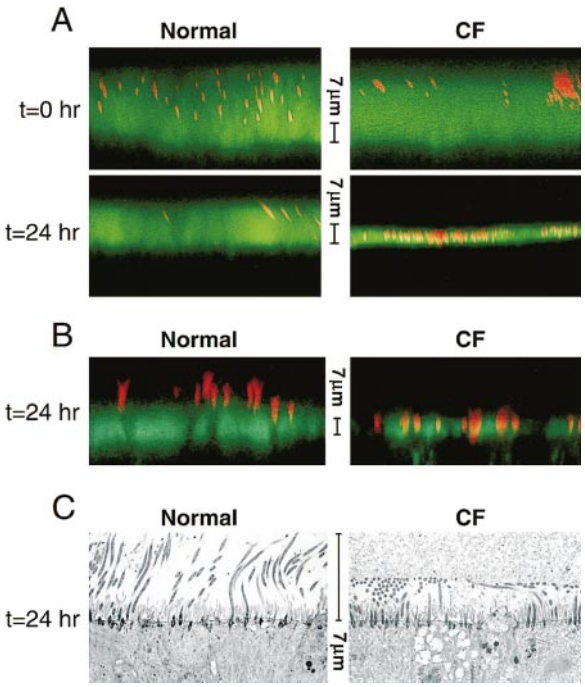

Figure 7. Airway Surface Liquid Topography in Normal and CF Planar Cultures

Note the calibration bar depicting PCL height (~7 μm) interposed vertically between each set of images.

(A) Localization of mucus and PCL in normal (Nl; left panels) and CF (right panels) cultures. Fifty microliters of PBS containing Oregon Green–dextran and 1 μm red fluorescent microspheres were added to culture surfaces and imaged by x-z scanning confocal microscopy. At t = 0 hr, both Nl and CF exhibited a zone free of beads (~7 μm—see scale bar), the PCL, and a zone marked by beads, the mucus layer. At t = 24 hr, the PCL and mucus zones were still discrete in the normal cultures, whereas the overall height of the ASL on CF cultures was greatly reduced, beads invaded the PCL, and the PCL height was diminished below the 6–7 μm usually observed. The x-z confocal images are representative of four individual experiments for both normal and CF cultures.

(B) Localization of mucus (red 1 μm beads) and cilia in normal (left panels) and CF (right panels) cultures. At t = 0 hr, 50 μl of PBS containing 1 μm red fluorescent beads and 0.5 μg/ml of fluorescein-conjugated wheat germ agglutinin (to label cilia) was deposited on culture surfaces. The x-z confocal images shown are representative of images obtained 24 hr later from three normal and CF cultures.

(C) Fine structure of mucus/PCL interface in normal (left) and CF (right) cultures. Normal and CF cultures (from Figures 6 and 7A) were fixed at 24 hr with PFC/OsO_4 and examined under low-power EM. Representative EMs from each tissue group are shown.

layer, cilia appeared trapped, unable to extend and supply the motive force for mucus transport. Thus, we conclude that the cause of slowed mucus transport in CF is depletion of the periciliary liquid compartment, likely complicated by thickening of the overlying mucus.

Discussion

Elucidation of the pathogenesis of CF lung disease requires an understanding of normal airway defense mechanisms against infection and how missing CFTR function(s) perturb these mechanisms. Our model studies have tested the roles of airway ion transport in the regulation of ASL ionic composition and tonicity, volume transport, and mucus transport, and investigated the impact of mutations in CFTR on these putative defense mechanisms.

ASL Tonicity as a Test of the Hypotonic ASL Hypothesis: Normal and CF ASL Are not Different, and Both Are Isotonic

To promote defensin activity, ASL must have a [NaCl] of <50 mM, a solution that would be significantly hypotonic (~100 mOsm/L) (Goldman et al., 1997). Using a lavage technique with independent measurements of Na^+, Cl^-, and H_2O by isotopic equilibrium, Zabner et al. (1998) calculated a [NaCl] for normal ASL of ~50 mM. Our direct chemical measurements of undiluted ASL samples from both planar and BF cultures, however, provided no evidence that normal airway epithelia produce an ASL that is hypotonic or low in NaCl (Figure 2A). Additionally, measurements of [Cl^-] directly in the ASL covering normal cultures with ion-selective microelectrodes confirmed "isotonic" Cl concentrations during volume absorption (Figure 6).

The observation that normal ASL is isotonic (Figure 2) is consistent with reports of relatively high water permeabilities for human and other mammalian airway epithelia (Farinas et al., 1997; Davis et al., 1998) and indicates that it is unlikely that airways function like sweat ducts to absorb salt across a water-impermeable epithelium. Two sets of experiments (Figures 3A and 3B) demonstrated that airway epithelia do not long maintain transepithelial osmotic gradients (>10') even when hypotonic solutions are added to airway surfaces. Similar kinetics of equilibration to isotonicity have been reported for hypotonic liquids added to human nasal surfaces in vivo (Johnson et al., 1998), suggesting that both in vitro and in vivo airway epithelia are too water permeable to produce or maintain hypotonic ASL.

With respect to modification of ASL by "surface forces," our morphologic studies revealed that the tips of cilia are covered by a mucus layer (Figures 4A and 7A). These observations parallel those from in vivo studies (Sims and Horne, 1997) and are inconsistent with formation of small radius menisci and high surface tensions at ciliary tips (Widdicombe, 1997). Using BFs plugged at both ends to simulate hydrostatically the effects of a meniscus or other "surface forces" (equal to >3600 cm H_2O if ASL [NaCl] = 50 mM), we detected no functional correlate of "surface forces" on ASL composition (Figure 4B).

Recently, Zabner et al. (1998) proposed an alternative to their suggestion that meniscus-associated capillary forces balance the osmotic pressure of low [NaCl], hypotonic ASL. They suggested that normal ASL could be an isotonic solution composed of low [NaCl] and "unidentified" osmotic material. Given the 50 mM ASL [NaCl] reported in their study (osmolality was not measured), approximately 200 mOsm/L of "unidentified" osmoles would be required. However, direct measurements of both osmolality and [NaCl] revealed that NaCl constituted the major fraction of the measured osmoles in normal ASL (Figure 2A). Thus, normal ASL in vitro does not contain a significant quantity of "unidentified" osmoles, a finding similar to that from in vivo studies of ASL composition (Boucher, 1994a).

We conclude that ASL in normal subjects is isotonic, with a [NaCl] similar to plasma and in a range that would hinder defensin activity (Figure 2A). This conclusion is consistent with the large body of ASL compositional data from experimental animals in vivo (Boucher, 1994a) and the most recent in vivo studies of ASL ionic composition from normal subjects (Knowles et al., 1997; Hull et al., 1998). Thus, it is unlikely that the hypotonic (low salt) ASL/defensin hypothesis describes normal lung defense. Our finding that the ASL covering CF airway epithelia in vitro is also isotonic (Figure 2B), consistent with recent in vivo data from uninfected CF subjects (Knowles et al., 1997; Hull et al., 1998), argues against a role in CF for ASL hypertonicity in disease pathogenesis (Gilljam et al., 1989).

Volume Absorption as a Test of the Isotonic Volume Absorption Hypothesis: Normal Airway Epithelia Are Volume Absorbing and CF Hyperabsorbing

If normal airway epithelia do not regulate ASL tonicity, do they regulate ASL volume? Jiang et al. (1993) reported that normal airway epithelia absorb liquid from large volumes placed on cultured airway surfaces. Others studied this question with smaller volumes of liquid added to culture surfaces to simulate the "thin film" of ASL in vivo (Smith and Welsh, 1993). These authors reported normal airway epithelia absorb little. We measured significant volume absorption by normal airway epithelia in BFs ("large volume" conditions) and across planar preparations ("thin film" conditions) with independent techniques (Figures 5 and 6A). Consequently, we conclude that normal human airway epithelia do absorb volume from the ASL compartment and that the absorptive process is isotonic (Figures 2 and 6B) and Na^+ dependent (Figure 5), as reported for freshly excised airways (Boucher, 1994a). These conclusions are consistent with predictions of thermodynamic models of airway epithelia based on ion and water permeabilities reported for human airway epithelia (Novotny and Jakobsson, 1996).

If mutations in CFTR do not perturb the ionic composition of ASL, do they perturb the rates of isotonic volume absorption across CF airway epithelia under "thin film" conditions? Jiang et al. (1993) reported that under conditions with large luminal volumes, volume absorption was increased in CF compared to normal airway epithelia, but others, using "thin film" techniques, did not detect this difference (Smith et al., 1994). In separate protocols, we found that CF airway epithelia absorb ASL isotonically from "thin films" at a rate significantly greater than normal airway epithelia (Figures 6 and 7). The abnormal rate in CF could reflect a defect in a "sensor" for ASL height or a problem in the "effector" elements in CF epithelia that modulate height, for example, accelerated Na^+ transport and/or the inability to secrete Cl^-.

CF ASL Volume Hyperabsorption Deranges Mucus Transport, in Part by Depletion of PCL

In the same planar preparations used to measure volume absorption, we asked if abnormal CF volume transport perturbs mucus transport? In two experiments (Figures 6 and 7), the accelerated absorption of ASL in CF cultures was associated with abolition of mucus transport at 24 hr, whereas mucus transport persisted in normal cultures.

The confocal studies of ASL volume, ASL topography, and mucus transport in living cultures and the EMs from PFC/OsO_4-fixed preparations revealed that cilia of normal epithelia beat in a well-defined PCL layer that is covered by a well-organized mucus layer (Figures 4A and 7A). The abnormally high rate of volume absorption in CF airway epithelia depleted the PCL layer, trapped cilia, and abolished mucus transport. Both confocal (Figures 7A and 7B) and EM studies (Figure 7C) demonstrated that the absence of mucus transport was associated with the penetration of mucus into the depleted PCL compartment. The measurements of Cl^- concentrations verified that the residual PCL, too shallow to support mucus transport, remained isotonic. The importance of the PCL height as a determinant of mucus transport was predicted by theoretical studies (Blake, 1975).

Relationship to Other Models and In Vivo Conditions

Zabner et al. (1998) recently reported a low [NaCl] in normal ASL and a reduced capacity for volume absorption by CF cultures. Differences in culture conditions could explain the apparent discrepancies between those data and ours. For example, Zabner et al. focused on conditions that produce cultures with high resistances to passive ion flow, which may function more like "tight" epithelia than the "moderately leaky" physiology representative of intact tissues (Boucher, 1994a). We focused on producing cultures that recapitulated a spectrum of functions of airway epithelia, including mucus transport. These cultures exhibited the following properties of intact airway epithelia (Wanner et al., 1996; Widdicombe, 1997): (1) high density of cilia per cell, measured by the interciliary distance ($\sim$0.1 μm; Figure 7C), suggesting that the radius of potential menisci formed at the tips of outstretched cilia would be the same as in vivo; (2) characteristic ciliary length ($\sim$7 μm; Figure 7C); (3) large fraction of the surface covered by ciliated cells (60%–80%; Figure 1); (4) rapid ciliary beat (10 Hz; C. W. D. et al., unpublished data); and (5) vectorial mucus transport (40 μm/s; Figure 6C), reflecting the integrated activity of cilia, periciliary liquid, and the overlying mucus gel, as occurs in vivo. Based on these comparisons, we believe that our model accurately mimics the spectrum of these and other properties of the epithelium in vivo, including surface forces.

However, regardless of criteria, no one culture system can totally represent all the functions of the complex epithelia that line airways. Thus, a comprehensive approach requires the use of complementary culture systems and comparisons between data derived from cultures and airway surfaces in vivo with identical techniques, for example, ion-selective electrodes (Knowles et al., 1997).

"Multiple Hit" Failure to Clear Thickened Mucus from CF Airway Epithelia

In CF cultures, the accelerated absorption of ASL produced a shallow PCL that likely also resulted in concentrated, or thickened, mucus. Cilia-dependent mucus

transport may also, therefore, be reduced because of abnormal biorheologic properties of concentrated mucus (Puchelle et al., 1995). Studies that controlled for particle deposition have detected reduced mucociliary transport in adolescents and young adults with CF in vivo (Regnis et al., 1994). However, the mammalian lung has "back-up" mechanisms to clear retained mucus when cilia-dependent clearance fails. Cough efficiency is theoretically directly proportional to ASL height and inversely proportional to ASL viscosity (Scherer, 1981), and experimental studies have emphasized the requirement for PCL as a "lubricant" for cough (Zahm et al., 1989). The reduced efficiency of "cough clearance" in young CF patients (Kohler et al., 1986) is consistent with reduced ASL/PCL height. Reduced ASL/PCL height and increased mucus viscosity are similarly predicted to reduce the efficiency of airflow-dependent clearance mechanisms in peripheral airways (Blake, 1975). As a consequence of "multiple hits" on virtually all mucus clearance mechanisms, we predict that concentrated mucus is retained on epithelial surfaces in vivo as plaque-like masses (Figure 6C).

The Role of Retained, Thickened Mucus in Pathogenesis of CF Airways Infection

The isotonic volume hyperabsorption hypothesis predicts that mucus retention precedes infection, whereas the hypotonic theory predicts that infection precedes mucus/inflammatory mass retention in CF. Perhaps the most compelling clinico-pathologic data to discriminate between these theories are those of Zuelzer et al. (1949), which demonstrated that retention of mucus (without inflammatory cells) in bronchioles was the earliest pathologic lesion in the airways of CF neonates dying within 48 hr of birth.

The hypothesis that retained mucus plaques/plugs serve as the nidus (surface) for the early and ultimately chronic CF bacterial lung infection is also consistent with recent reports of the pathogenesis of airways bacterial infection. The initial bacterial infection of airway explants occurs in the mucus rather than epithelial cell compartment of airway surfaces (Ulrich et al., 1998), and bacteria in airway mucus can assume a biofilm morphology (Tsang et al., 1994) similar to that in lungs of CF patients (Lam et al., 1980). Thickening of mucus may also reduce the activity of microbiocidal substances in this compartment and add to the problem of poor clearance. We cannot definitively account for the high prevalence of *Pseudomonas aeruginosa* infections in CF airways (Davis et al., 1996), but several laboratories have demonstrated selective attachment/growth of *Pseudomonas* over other non-CF respiratory pathogens on surfaces comprised of human airway mucus (Vishwanath and Ramphal, 1984; Nelson et al., 1990).

We speculate that once the initial infection of adherent mucus develops, perhaps triggered by neonatal infection/aspiration–induced mucin hypersecretion, bacterial exoproducts derived from the biofilm damage the epithelium, induce bacterial adhesion to epithelia (Plotkowski et al., 1992), unleash an abnormally large inflammatory response (Konstan and Berger, 1997), and ultimately produce bronchiectasis (Davis et al., 1996).

Comparison of CF Pathogenesis to Other Genetic Diseases Affecting Airways Defense

Our data also may explain the differences in the phenotype between CF and other genetic diseases associated with abnormalities in airways defense. The hypotonic (low salt) ASL hypothesis predicted that patients with pseudohypoaldosteronism (PHA), with loss-of-function mutations in the epithelial Na^+ channel (ENaC) that mediates Na^+ airway epithelial transport, should have isotonic ("high salt") ASL and a syndrome like CF (Quinton, 1994). Patients with PHA have isotonic ASL (Kerem et al., 1997), as do normal subjects (Knowles et al., 1997; Hull et al., 1998), but do not exhibit a syndrome of CF-like infectious lung disease (Kerem et al., 1997). Rather, as predicted by the isotonic volume hypothesis, PHA patients have an excess of ASL volume due to the absence of Na^+-dependent volume absorption. Data from patients with gain-of-function mutations in ENaC, Liddle's syndrome, could also be informative. However, it is not yet clear whether Na^+ transport in the respiratory tract is raised in these patients (Baker et al., 1998; Stutts et al., 1998).

In primary ciliary dyskinesia (PCD), there is absent basal mucociliary clearance due to abnormal ciliary beat. PCD exhibits a milder phenotype than CF, with patients often not experiencing significant lung disease until adulthood (Levison et al., 1983). However, cough clearance is well maintained in PCD (Noone et al., 1996), likely reflecting adequate ASL hydration and preservation of PCL. We speculate that the "multiple hits" on all mucus clearance mechanisms in CF explain the more severe phenotype in CF lung disease as compared to PCD, in which there is a selective loss of cilia-dependent mucus clearance.

Integrated View of ASL Homeostasis in Health and Disease

Our study, coupled with recent studies of culture systems and in vivo data, leads to an integrated view of normal ASL physiology and its relationship to CF pathogenesis. In brief, in the normal lung, the entire ASL (both the PCL and mucus layers) sweeps up the airway surfaces (Matsui et al., 1998). Airways absorb salt and water isotonically to adjust the volume/height of the ASL components, for example, PCL, to maintain efficient mucus clearance as liquid converges proximally. Consequently, a major role of the airway epithelium in airways defense is isotonic volume transport rather than modulation of airway surface tonicity.

In CF, excessive isotonic volume absorption locally reduces PCL and concentrates mucus on airway surfaces. Whereas we speculate that this is the major defect in CF airways, we do not rule out the contributions of reduced isotonic volume secretion from submucosal glands (Boucher, 1994a) or of reduced secretion of isotonic liquid at the sites in the distal lung that produce the liquids that move up airway surfaces. These secretory defects would only exacerbate the imbalance of secretion/absorption that hinders airway mucus clearance and, possibly, impair the delivery of microbiocidal activities to airway surfaces.

Therapeutic Implications

Finally, our studies have implications for therapeutic strategies for treating CF lung disease. For example, if

the hypotonic (low salt) ASL/defensin hypothesis were correct, a major therapeutic goal would be to design therapeutic agents that remove salt but not water from CF airway surfaces. In contrast, our data strongly support the isotonic/volume absorption hypothesis (Figures 6 and 7). Here the goal is to add isotonic liquid (salt and water) to airway surfaces to restore the volume of PCL layer and the normal viscoelastic and adhesive properties to the concentrated mucus. Our observation that addition of isotonic NaCl solution to CF cultures with impacted mucus transport rapidly restores transport argues that this type of therapy may be successful and should be initiated early in the natural history of this disease.

Experimental Procedures

Human Airway Epithelial Cell Isolation and Culture

Human airway epithelial (HAE) cells were obtained from freshly excised bronchial specimens from CF and normal (Nl) subjects, cells disaggregated by protease digestion (Matsui et al., 1998), and, as primaries or after expansion on tissue culture plastic (P1 cells), cultured using two systems. For planar culture preparations, the cells were seeded on Transwell Col membranes (12 or 24 mm diameter, Costar) in modified BEGM media under "ALI conditions" until fully differentiated (~4 wk; see Figure 1). For tubular cultures, cells were seeded in the lumen of hollow fibers (Grubb et al., 1997) and maintained in modified BEGM media until study (~2 wk; see Figure 1). Planar preparations with R_ts $> 300\ \Omega\ cm^2$ and BFs with PDs $>$ 2 mV (R_ts were not measured) were studied. For combined studies of mucus and volume transport, planar preparations were screened for rotational mucus transport by addition of 20 μl of 0.02% volume fluorescent microsphere suspension (carboxylate modified, 1 μm diameter, red fluorescence, Molecular Probes, Eugene, OR) to the apical surface.

Humidified Incubator

Planar preparations were incubated in a specially humidified incubator. Humidified air within the incubator was recirculated by an air pump (SA-X5, Penn-Plax, Garden City, NY) through the inner sleeve of water vapor–permeable tubing (Nafion, Perma Pure, Toms River, NJ) whose outer sleeve was filled with H_2O. Wet gauze pledgets were inserted under the Costar lids. Evaporative water loss was assessed by: (1) change in fluorescence of an aqueous solution of Oregon Green–dextran (200 μl; 0.1 mg/ml) deposited in a well of a 6-well tissue culture plate, and (2) the change in the diameter (and calculated volume) of a 500 nl droplet deposited on the bottom of a 6-well tissue culture plate.

Measurement of Airway Surface Liquid Osmolality and Ionic Composition

For the planar preparation, the culture was washed three times with isotonic phosphate-buffered saline (PBS [in mM]: $Na^+ = 150$, $K^+ = 4.2$, $Cl^- = 140$, $PO_4^-/PO_4^{2-} = 9.0$), PBS aspirated thoroughly, PBS (200 μl) added to the surface of a 24 mm (4.5 cm^2) planar preparation, a 2 μl sample obtained at t = 0, and the preparation placed in the humidified incubator. At 24 hr, a constant-bore microcapillary pipette was lowered onto the surface of the preparation with a micromanipulator through a small-bore hole drilled in the top of the T-Col lid to sample ASL.

For the BF preparation, the BF was plugged at one end, and the other end was connected to a constant-bore capillary filled with Krebs bicarbonate Ringer (KBR). BFs were immersed in KBR at 37°C in an incubator and, after 5 or 24 hr, the luminal contents expelled as a droplet under water-saturated mineral oil, following which the droplet was drawn into a microcapillary tube between oil columns.

Samples (4–8 μl) from microcapillaries were deposited on filter paper discs and osmolality measured with a vapor pressure osmometer (Vapro 5520, Wescor, Logan, VT). For measurements of Na^+ or Cl^- concentrations, the sample volume was measured from the length of the liquid column in the capillary tube, the contents diluted into ultrapure 0.1 N nitric acid, and measurements of Na^+ made by flame emission spectroscopy and Cl^- by amperometric titration, as previously described (Knowles et al., 1997). The variance of these measurements was less than 5% (Knowles et al., 1997).

The Cl^- concentration of ASL in the planar preparations was measured in situ using double-barreled Cl^--selective microelectrodes (Willumsen et al., 1989). The microelectrode was positioned with a micromanipulator in the liquid covering the culture preparations, stable measurements ($>$ 15 s) recorded, and values determined from standard calibration curves.

Measurements of Volume Absorption

For planar preparations, volume absorption was measured from the change in concentration of an impermeant solute, blue dextran (BD, 2%). The samples (~3–6 μl) collected at t = 0 and t = 24 hr were assayed for BD as previously described (Ballard and Gatzy, 1991). For BFs, the rate of volume flow was measured using BFs with attached microcapillaries by monitoring the movement of the meniscus within the capillary hourly for 5 hr (Grubb et al., 1997). For studies of the role of Na^+ in volume absorption, Na^+ was replaced with NMDG (Grubb et al., 1997).

Optical Measurements of Volume Absorption, Mucociliary Transport, and Airway Surface Liquid Topography

Optical measurements of volume absorption and mucus transport were made in CF and Nl planar (12 mm T-Col) cultures in two sets of experiments. In the first, all cultures derived from six consecutive Nl and CF specimens that exhibited rotational mucus transport and $R_t > 300$ (~25% of cultures, i.e., two to three cultures/specimen) were studied for volume absorption, Cl^- concentration, and mucus transport (Figure 6). In the second, all cultures with the above properties generated from four consecutive Nl and CF patients (two to three cultures/specimen) were used for studies of volume absorption and PCL height (Figure 7). For each study, the ASL was labeled at t = 0 with 50 μl of a fluorescent probe suspension in PBS, consisting of a fluorophore conjugated to 10,000 MW dextran (Oregon Green or Texas Red, 2 mg/ml, Molecular Probes, Eugene, OR) and 1 μm fluorescent beads (red or green, 0.02%, Molecular Probes). To avoid evaporation, studies of mucus transport, which did not require opening the Costar lid, were made first. After these measurements, the lid was removed, a thin layer of mineral oil, or for repetitive measures, PFC, added to the surface of ASL, and optical sections obtained within 10 min.

Measurements of Mucus Transport Rates

Mucus transport rates over the surface of planar cultures were measured from images (5 s exposures) acquired with an inverted epifluorescence microscope (Leica DMIRB, Wetzler, Germany) and a CCD camera (Hamamatsu C5985, Hamamatsu, Japan). The linear velocity of bead transport was measured at a 1 mm distance from the center of rotation based on least-squares fits to the data (Matsui et al., 1998).

Height of Airway Surface Liquid

ASL thickness was measured at five predesignated points (center and four spots between the center and the edge). Images perpendicular to the ASL layer were acquired as x-z sections with the confocal microscope (Leica) and analyzed with an image analysis system (MetaMorph, Universal Image Co., West Chester, PA) with the observer blinded to tissue code. The discrepancy between the predicted height increment for a 50 μl volume added to a 1 cm^2 culture and the measured values reflects accumulation of liquid at the edge between the culture and the wall of the T-Col insert. For studies of Na^+ dependence of volume absorption, the probes (Oregon Green–dextran, 1 μm beads) were added in PBS or Na^+-free media (Na^+ replaced by NMDG). For analyses of the effects of hypotonicity, the probes were added in a 2/3 dilution of PBS with water (100 mOsm).

Morphologic Studies

To study the topology of the ASL, we utilized a nonaqueous fixative that preserves airways mucins in situ (Sims and Horne, 1997). A 1% solution of OsO_4 dissolved in a perfluorocarbon (FC-72, 3M Co.) was added gently to the top of the cultures. After fixation, samples were directly immersed in 100% ethanol, processed for epon/araldite resin embedding using conventional procedures, and 1 μm thick

and ultrathin sections prepared for light and electron microscopy, respectively.

Statistics

N represents the sample (donor) size, typically two to three specimens from each donor subject. We found no differences between results from primary and P1 cell preparations from donors; consequently, these data were grouped for comparison. Comparisons were made with Student's t test or, where appropriate, the Mann-Whitney U test, with $p < 0.05$ considered significant. Unless otherwise stated, all data reported are mean $\pm$ SE.

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