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Site of Fluid Secretion in Small Airways

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Abstract

The secretion and management of readily transportable airway surface liquid (ASL) along the respiratory tract is crucial for the clearance of debris and pathogens from the lungs. In proximal large airways, submucosal glands (SMGs) can produce ASL. However, in distal small airways, SMGs are absent, although the lumens of these airways are, uniquely, highly plicated. Little is known about the production and maintenance of ASL in small airways, but using electrophysiology, we recently found that native porcine small airways simultaneously secrete and absorb. How these airways can concurrently transport ASL in opposite directions is puzzling. Using high expression of the Na-K-2Cl cotransport (NKCC) 1 protein (SLC12a2) as a phenotypic marker for fluid secretory cells, immunofluorescence microscopy of porcine small airways revealed two morphologically separated sets of luminal epithelial cells. NKCC1 was abundantly expressed by most cells in the contraluminal regions of the pleats but highly expressed very infrequently by cells in the luminal folds of the epithelial plications. In larger proximal airways, the acini of SMGs expressed NKCC1 prominently, but cells expressing NKCC1 in the surface epithelium were sparse. Our findings indicate that, in the small airway, cells in the pleats of the

epithelium secrete ASL, whereas, in the larger proximal airways, SMGs mainly secrete ASL. We propose a mechanism in which the locations of secretory cells in the base of pleats and of absorptive cells in luminal folds physically help maintain a constant volume of ASL in small airways.

Keywords: airway epithelium; airway surface liquid; bronchi; bronchioles; cystic fibrosis

Clinical Relevance

This work provides the first histological basis for a new concept to explain how airway surface liquids are maintained in small airways by separate cells in the pleats and folds of the plicated luminal epithelium that concurrently secrete and absorb. The mechanism of fluid maintenance was not understood previously, and this explanation should improve the ability to understand normal function as well as pathologies in diseases of small airways, such as cystic fibrosis, in which fluid transport is disrupted.

The small airways of the respiratory tree present a unique anatomical architecture that appears in cross-section as circular tubes, the luminal epithelium of which is plicated into folds and pleats (Figure 1). The purpose or function of this structural design is generally thought to allow the diameter of the airway to expand and collapse during inhalation and exhalation,

respectively, without a change in the surface area of the airway lumen. Even so, all airway surfaces must be bathed continuously by a liquid layer that is crucial to clearing pathogens and debris, including mucus secretions from the airway lumens (1–3). The volume or thickness of the airway surface liquid (ASL) necessarily depends on both

secretory and absorptive fluid transport activities (4–6), and impairment of the management of ASL may lead to pathology, such as cystic fibrosis (CF) (7–11) and possibly other lung diseases (12). The ASL of larger proximal airways of the respiratory tract can be supplied by secretory epithelial cells of submucosal glands (SMGs) (13–15). However, in

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Figure 1. Plicated structure of lumen of small airway. Cross-section of small distal airways used for immunohistochemistry analysis (A). Airway stained with hematoxylin illustrates the accordion-like, plicated structure of the lining epithelium forming “folds” and “pleats” regions (black arrows). Scale bar, 100 μ m. (B) A higher magnification of region of pleats and folds of small airway stained with hematoxylin and periodic acid-Schiff to identify goblet cells by the presence of mucin (purple-magenta) associated with the basal domain of pleats (examples: white arrows). Scale bar, 25 μ m.

smaller, more distal (conducting bronchiolar) airways, SMGs are absent, presumably for economy of respiratory volume and pulmonary compliance, so that the luminal surface epithelium must be capable of both secretory and absorptive functions to maintain an optimal volume of ASL (4, 5).

We recently found that freshly isolated segments of native small airway epithelia simultaneously secrete and absorb (5). This finding requires that separate secreting and absorbing epithelial cells be present and functioning independently, but the sites or domains of secretion and absorption in the epithelial lining is not known. By analogy with the intestine (16), we considered that, similar to intestinal crypts, secretory cells might occupy the basal domain of pleats, and that, similar to intestinal villi, absorptive cells might occupy the luminal domain of folds in the plicated surface epithelium of the airway (Figure 1).

To evaluate this hypothesis, we looked to the expression and distribution of Na-K-2Cl cotransport (NKCC) 1 in the airways of the pig as a model of human lung (17), as high expression of the NKCC1 (SLC12a2) is a well characterized marker for fluid-secreting cells (18–21). Specialized fluid (or Cl[−])-secreting cells, like those found in the acini or crypts of exocrine glands or intestine, characteristically express at least 20-fold more NKCC1

protein than nonepithelial cells or absorptive epithelial cells (22). In addition, high expression of NKCC1 is not only a selective marker for fluid secretory cells, but, in fluid-secreting cells, it is highly expressed only in the basolateral, not in the apical, cell membrane. We observed this pattern of intense immunofluorescence labeling of NKCC1 only in cells located in the contraluminal (basal) domain of the pleats of the epithelial lining of the small airway and in SMGs of the proximal large airway.

Although we have not similarly characterized sites for absorption, these results suggest that ASL is secreted from fluid-secretory cells within the pleats and absorbed by cells on the folds of the plicated distal airways, whereas, in larger, proximal airways, ASL would appear to be predominantly secreted from glands and absorbed by the luminal surface epithelium.

Materials and Methods

Whole lungs were resected intact from farm pigs (>30 kg) killed at a local abattoir and immediately transported and maintained under ice until used within 1–3 hours with University of California–San Diego Institutional Animal Care and Use Committee approval. Airways were dissected in chilled Ringer solution (4–10°C) as described previously (5).

RT-PCR Analysis

RT-PCR analysis for the expression of NKCC1 protein was performed on freshly isolated segments of small airways. Total RNA was purified and assayed as described previously (23). From the published sequence in GenBank/Primers, the sequence of primers used to identify the expression of NKCC1 (accession no. XM_003123899) were designed as forward 5' TCCAGGTGATGAGCATGGTGTCTAG and reverse 5' GTTAAGATGTAGCCACGAAAAGGT. Primers for β -actin (accession no. AY550069) were forward 5' TCAACTCATCATGAAGTGCAC and reverse 5' AAG AAGAGT CCGCTAG AAGCATT.

Western Blot Analysis

Isolated small airways were extracted and homogenized in cold lysis buffer in the presence of proteases inhibitors. Extracted proteins were separated by 10% SDS-PAGE under nonreducing conditions and electrotransferred onto Immobilon-P membrane (Millipore, Bedford, MA). Transferred proteins were labeled with rabbit antibodies against the amino (antibody N-terminal domain [NT]) or carboxy (antibody C-terminal domain [T84]) terminal domains of human NKCC1 (24) and detected with an alkaline phosphatase-conjugated secondary antibody (Sigma, St. Louis, MO).

Immunohistochemistry Analysis

Small specimens of peripheral lung (~ 0.4 cm³) or trachea (~ 0.25 cm³) were dissected, embedded in freezing media, immediately frozen in isopentane at -80° C, and stored at -80° C. Frozen specimens were sectioned (~ 5 – 7 μ m) at -20° C and thaw-mounted on glass slides (Fisher Superfrost/Plus, Pittsburgh, PA). Before labeling, sections were dehydrated in -20° C acetone for 5 minutes, treated with 1.5% sodium borohydride in PBS for 20 minutes to reduce autofluorescence, and then blocked with 3% BSA in PBS for 30 minutes. Sections were incubated with primary antibodies against NKCC1 (NT or T84 [24]), Mucin 5AC (MUC5AC) (Invitrogen, Carlsbad, CA), or anti-zonula occludens-1 (ZO-1) (Zymed; Invitrogen) for 2 hours at 4° C, followed by the appropriate secondary antibodies (Alexa Fluor 488 goat anti-rabbit and Alexa Fluor

555 goat anti-mouse; Life Technologies, Eugene, OR) for 1 hour. After four rinses with PBS, nuclei were stained with 4',6-diamidino-2-phenylindole (Roche, Indianapolis, IN) and specimens were mounted with antifade reagent. Confocal images were digitized with IP Labs software (BD Bioscience, San Jose, CA). Images were visualized individually or assembled as a collage. Antibody specificity was verified by processing paired sections without primary antibody (Figure E4).

Morphometric Analysis of Cell Distributions

Images of NKCC1-labeled airway cross-sections were imported into MetaMorph software (Molecular Devices, Sunnyvale, CA) and analysis was performed on 56 randomly selected plicated regions in small airways from 7 different animals to measure the relative positions of the nuclei of NKCC1-positive and NKCC1-negative cells along the pleat-fold axis from the contraluminal base of the pleat (0%) to the luminal tip of the fold (100%). A *P* value less than 0.01 was taken as indicating significantly different mean distances of the two groups of cells from the base of the pleat.

Results

NKCC1 Expression in Porcine Small Airway Tissue

The expression of NKCC1 in porcine small airways was confirmed by RT-PCR (Figure E2A) and Western blot analysis (Figure E2B). The labeled band migrated at approximately 170 kD, consistent with NKCC1.

NKCC1 Localized in a Subset of Epithelial Cells in the Small Airway

Cross-sections of porcine small airways showed that the lining epithelium has a plicated, accordion-like structure forming "folds" and "pleats" (Figure 1). Characteristically, NKCC1 is highly expressed in the basolateral membrane of fluid-secreting epithelia cells. The distribution of NKCC1 in these airways was assessed by confocal immunofluorescence microscopy using antibodies directed against the NT or the T84. Both antibodies prominently labeled an identical domain of epithelial cells, designated as NKCC1 high expresser cells.

Another domain of epithelial cells showed comparatively weak or undetectable NKCC1 labeling, designated as NKCC1-negative cells (Figures 2 and 3). Similar distributions occurred in multiple specimens from different small airways (Figures 3; *see also* Figures E2 and E3 in the online supplement). In the absence of primary antibody, no significant labeling was observed (Figure E4).

Morphometric analysis confirmed a distinct distribution of NKCC1 expression along the fold-pleat axis with high expresser cells clearly more abundant near the basal domain of the pleats, whereas cells not staining positive for NKCC1 were more abundant in the luminal domain of the folds (Figure 3). The distributions of labeled cells versus unlabeled cells were highly significantly different (Figure 3; *P* < 0.0001).

Expression of NKCC1 in Proximal Large Airway

To demonstrate and verify that high expression of NKCC1 correlates with a known secretory phenotype (e.g., gland acinar cells), specimens of porcine trachea were immunolabeled for NKCC1. In the large airway, intense NKCC1 labeling, at intensities similar to those in small airway high NKCC1-expressing cells, was detected predominantly in acinar SMG acinar cells (Figure 4, Figure E5),

although occasional NKCC1 high expressing cells were observed in some (Figure 4), but not other, sections of the surface of the tracheal epithelium (Figure E5).

Discussion

The layer of liquid on the surface (ASL) of bronchi and bronchioles is intrinsic to pulmonary hygiene, and its volume must be closely managed by the airway epithelium to prevent obstructing air flow by either flooding the airway, due to excessive fluid secretion, or by blocking the airway with desiccated debris, due to excessive fluid absorption. However, native epithelial cells do not physiologically absorb and secrete simultaneously. That is, in the intestine, deep crypt cells secrete, whereas surface and villus cells absorb fluids; in exocrine glands, acinar cells secrete precursor fluid, whereas ductal cells absorb solutes (e.g., sweat glands, salivary glands, pancreas, etc.). In our preparations here from the pig, SMGs were rarely observed, indicating that these porcine airways (~1-mm diameter) are bronchiolar rather than bronchial, and, as such, probably correspond to small airways arising at about the eighth–ninth generation in humans (25). Similar to the luminal surface of the pig airway in cross-section (Figures 1–3), the proximal human bronchiolar airway is also plicated, giving

Figure 2. Na–K–2Cl cotransport 1 (NKCC1)-positive cells in the luminal lining of small airway. Immunolabeling of a histological cross-section of a small airway identifies subsets of epithelial cells with strong NKCC1 labeling (green, examples: arrows) mainly located toward the base of the pleats in the plications of the epithelium. Scale bar, 100 μ m. DAPI, 4',6-diamidino-2-phenylindole.

Figure 3. Distribution of NKCC1-positive and NKCC1-negative cells in the epithelial lining of small airways. Images of small airways were analyzed with Metamorph software (Molecular Devices, Sunnyvale, CA) to quantify the frequency distribution of NKCC1-positive and NKCC1-negative cells. Micrographs of small airways (example, *left*) were used to measure the distribution of cells along the epithelial pleat-fold axis as a fraction of the distance between the bottom of the pleat and the top of the airway fold. The plots of distances (*right*) show that NKCC1-positive cells (green) were distributed predominantly in the first half of the distance between the bottom of the pleats and the top of the fold (mean $\pm$ SD = $25.0 \pm 21.9\%$), whereas NKCC1-negative cells appeared predominantly in the more luminal half of the distance to the tip of the airway fold ($72.5 \pm 29.1\%$; *** $P < 0.0001$).

rise to numerous pleats and folds that run axially along the lumen (5, 10, 26–31). This organization allows the small airway to be structurally and physiologically analogous to other secretory/absorptive systems that carry out secretion and absorption independently. To wit, because only the contraluminal domain of the pleats labeled almost exclusively for NKCC1, but not the luminal domains of the folds, the two domains of the epithelial plications appear to serve distinct functions (Figures 2 and 3, Figures E2 and E3).

Hence, if this tissue secretes and absorbs concurrently (5), we surmise that most of the epithelial cells of the luminal folds that do not express high levels of NKCC1 (Figures 3–5) are separate absorptive cells. We did not assay for specific cell types, except to demonstrate periodic acid–Schiff–positive mucus cells, but, at this level in the airway, the majority of epithelial cells are thought to be ciliated (32, 33), and because about half

of the cells labeled positive for NKCC1 (Figure 3), both secretory and presumed absorptive cells are likely ciliated. It is not clear at this level what role, if any, club (Clara) cells might play in fluid transport, as they are perhaps better characterized as mainly secreting electron-opaque protein granules as well as electron-translucent mucin granules (34), and may transform into either ciliated or mucus goblet (35); however, periodic acid–Schiff–positive cells that we took for mucus cells were common in the basal region of the pleats (Figure E3). These implications could suggest that ciliated cells may convert, as in the intestine, from secretory to absorptive transport phenotypes. Other than our previous results localizing the CF transmembrane conductance regulator anion channel (23), which was associated with the entire luminal surface of the bronchiolar epithelium within the pleats and over the folds, we are not aware of localization of any other ion transport components in

native small airways. The fact that CF transmembrane conductance regulator is well known to support both absorption and secretion (36), and is reported in ciliated cells (31, 37), leaves it well suited and compatible for both roles here. Nonetheless, it is virtually certain that the epithelial Na^+ channel is expressed in this tissue (23, 29) as it is in ciliated cells of the large airways (38). Clearly, this portion of the conducting airways awaits further and more definitive explorations of the numerous fluid and electrolyte transport components and systems that are undoubtedly present (33, 39).

Physiologically then, this apparent structural organization of secretion in the basal domain of the pleats and of absorption in the luminal domain of the folds in the plicated airway lumen favors local recycling of ASL fluid between the two domains (Figure 5), and renders the secreting pleats in the airway analogous to structures of other organs that secrete fluid in that solutes (electrolytes) are actively secreted into a small, diffusion-limiting compartment (i.e., a tubule, acinus, or crypt). The physical barriers of such compartments limit solute diffusion after electrolyte secretion to affect efficient iso-osmotic coupling of water for isotonic fluid transport (40). Here, like secretory units in glands, the opposed surfaces of small airway pleats create the constraining barriers of a space that restricts diffusion of secreted solutes for iso-osmotic coupling with water to secrete ASL. Secretions so formed emerge from the pleat space to bathe the surface of the folds of absorptive cells, which reabsorb, i.e., “recycle,” the ASL secretion back to the serosal fluid (Figure 5).

This view of the pleat for iso-osmotic coupling cannot be taken as a static structure, but rather as a unique, dynamic mechanism ideal for mixing and moving luminal fluids at the air–liquid interface of airways. That is, during exhalation, the diameter of the small airway contracts, thereby squeezing or partially squeezing the pleats together, which would force fluid out of the pleat space onto and over the folds in the lumen (Figure 5, *right*). During inhalation, on the other hand, the diameter of the small airway increases, spreading the pleats apart and increasing the inner pleat space for fluid accumulation and re-entry into the larger volume of the opened

Figure 4. Identification of NKCC1 in proximal large airway. Cross-section of tracheal wall immunolabeled NKCC1 (green) in cells of submucosal gland acini intensely (arrows). However, NKCC1 labeling in cells in the surface epithelium was observed in very few epithelial cells (examples, arrowheads). The luminal surface was delineated by zonula occludens 1 (ZO-1) immunolabeling of tight junctions (red). Nuclei were stained with DAPI (blue). Scale bar, 50 μm .

pleat (Figure 5, left). Consequently, the breathing cycle promotes continuous agitation of the ASL, preventing stasis and stagnation of debris and ensuring fluidity for mucociliary clearance. Complicit in this design, we note that mucin-secreting goblet cells normally are found more concentrated near the closed end of the pleat among the secretory NKCC1 high expressing cells (Figure 1B, Figure E3). The agitation of ASL that accompanies pleat movements seems well suited for the release, expansion, and mixing of mucins secreted with fluids within the pleat space for optimal transport along the luminal surface via the ciliated escalator to ensure airway hygiene.

In view of concerns regarding mechanisms to regulate the ASL layer (volume) (2, 41–45), it is tempting to surmise that the secreting-pleat/absorbing-fold structure may function toward autoregulation of the ASL volume in the small airway. That is, basal secretion in the pleats ensures that ASL is always present within the pleat space, where it cannot be reabsorbed, but always remains immediately proximal to absorbing tissue. Thus, even though absorptive capacity should normally exceed secretory activity, the fluid movements induced with the breathing cycle ensure continual wetting of the entire luminal surface, including the absorbing folds, as noted above, but because the folds absorb

secretions concurrently, fluids cannot accumulate excessively. Even though absorption apparently exceeds secretion (46), the mechanism obviates excessive fluid absorption, because pleats only secrete and cannot absorb fluid so that a constant, residual volume of ASL can be maintained in a stable steady state (Figure 5).

In contrast to small airways, large airways are replete with SMGs situated mainly between cartilaginous rings, which limit changes in airway diameter (47). Intriguingly, when we examined freshly excised tracheal tissue as a control for NKCC1 labeling specificity and as representative of large airways, we found intense labeling of gland acini, as expected for fluid-secreting cells, validating the antibody biomarker label for secretion. However, we found very little NKCC1 labeling of cells in the surface epithelium of the trachea. A few cells were labeled, but the overall paucity of labeled cells (Figure 4, Figure E5) suggests that proximal airway surface cells, as a group, are not secretory, and are probably essentially absorptive; the distribution of the NKCC1 labeling suggests that fluid is secreted predominately by the underlying SMGs, as assumed for modeling fluid recycling in bronchi (48). These results were somewhat unexpected, in view of several reports that the native tracheal surface epithelial cells secrete Cl^- and, therefore, fluid (43, 49–55). However, with their observation that Na^+ absorption and Cl^- secretion appeared to behave independently, separate, distinct cells were suggested to be responsible for each direction of transport in tracheal tissue as well (50), which then begs the question, “which cells in which direction?” Certainly, the abundance of submucosal secretory glands throughout the proximal airways also begs the question of why cells in the surface epithelium would secrete as well as absorb when glands are abundantly present that supply fluid to the surface where absorptive activity should limit excessive fluid accumulation on the airway. Among epithelial cells, the proximal airway surface cells are unique in transporting fluid at an air–liquid interface. If they, in fact, do achieve bidirectional transport, they also would be unique among epithelial cells as now known.

Figure 5. Illustration of continual agitation of airway surface liquid (ASL) in a small airway. During exhalation, pulmonary elastic recoil and compression contract the airway, compressing the space within the pleats and forcing ASL (blue) into the lumen. Upon inhalation, intrathoracic forces expand the airway diameter, increasing the volume within the pleats, allowing surface tension to return ASL to the pleat space. Conceivably, these repetitive actions stir and mobilize the fluid as a function of the breathing cycle. If fluid absorption toward the tips of the folds (red arrows) normally exceeds concurrent fluid secretion in the base of the pleats (blue arrows), the airway would still retain fluid and continue to be wetted without flooding or drying excessively.

In summary, NKCC1 antibody labeling as a selective marker for fluid-secreting cells indicates that the source of ASL secretion for bronchiolar airways

is in the contraluminal domain of pleats in the plicated epithelium of the airway. In large proximal airways, NKCC1 distribution indicates that the source

of ASL fluid is mainly from the underlying SMGs. The lack of NKCC1 labeling of the cells of the luminal regions of the folds of small airways and of the cells of the luminal surface of large airways suggests that these epithelial tissues are predominantly absorptive. Concurrent fluid secretion and absorption separated, respectively, to the contraluminal and luminal domains of the pleats and folds of the luminal surface in the presence of airway expansion-contraction during the breathing cycle continually wet and enhance cleaning the small airway surfaces with ASL, without flooding and without desiccating these small conducting airways. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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