Regulation of depth and composition of airway surface liquid

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ABSTRACT: We review the factors which regulate the depth and composition of the human airway surface liquid (ASL). These include secretion from airway submucosal glands, ion and fluid transport across the surface epithelium, goblet cell discharge, surface tension and transepithelial gradients in osmotic and hydrostatic pressure. We describe recent experiments in which we have used low temperature scanning electron microscopy of rapidly frozen specimens to detect changes in depth of ASL in response to submucosal gland stimulation. We also present preliminary data in which X-ray microanalysis of frozen specimens has been used to determine the elemental composition of ASL.

Keywords: Airway surface liquid, submucosal glands, active transepithelial ion transport, low temperature scanning electron microscopy, X-ray microanalysis

The airways are lined with a film of fluid, which is 5-20 μm deep in health. This fluid is believed to consist of two phases [1]. A watery fluid of low viscosity, the "periciliary sol" surrounds the cilia. Above this lies a viscous mucous gel. The cilia are able to beat in the sol. Their tips contact the underside of the mucous blanket and propel it towards the mouth. This system of "mucociliary clearance" serves to remove particles trapped in the mucous gel. The concept of two layers of airway surface liquid (ASL) has been supported by a variety of microscopical studies [2-5] (see Fig. 1).

Factors influencing the liquid content of sol and gel have been identified but their relative contributions are imperfectly understood. Water will move into the airway lumen in response to active Cl secretion across the epithelia of the tracheal surface and of submucosal glands. Active absorption of Na across the surface epithelium serves to remove liquid. These active ion transport processes move liquid by generating local osmotic gradients across the epithelium [6]. Hydrostatic pressure gradients across the epithelium, and differences in osmotic pressure between the media bathing the epithelium's two surfaces will also influence the net movement of liquid into or out of the lumen. Evaporation is an important factor in the upper airways. Finally, forces of surface tension generated by the closely packed cilia may serve to hold liquid in the airway lumen [7]. These factors are considered in turn below. Finally, we describe recent experiments in which we have used low-temperature scanning electron microscope (LT-SEM) to determine the depth and composition of the airway surface liquid (ASL).

Gland secretion

The adult human trachea has about 10 submucosal gland openings per mm^2 of airway surface [8]. It is unknown how the structure and numbers of glands changes down the airways, except that bronchi have glands, and bronchioles don't [14]. In each gland, a ciliated duct leads from the airway surface to an expanded collecting duct into which empty ~10 secretory tubules, each with multiple branches ending in closed acini [9]. The acini are lined by serous cells, the tubules by mucous cells [10]. It is believed that the serous cells secrete fluid which serves to flush out mucins released by the more proximal mucous cells [11]. Consistent with this hypothesis, gland serous cells contain much the highest levels of the cystic fibrosis transmembrane conductance regulator of any cell type in the airways [12]. This protein is an apical membrane Cl channel involved in transepithelial transport of Cl and water [13].

The secretory rate of human airway glands is not known. Maximal secretion from individual cat tracheal glands is ~10 nl/min [15, 16]. If this rate applies to human tracheal glands, then total gland secretion could be as high as 60 $\mu l.cm^{-2}.h^{-1}$. This would increase the depth of the ASL by ~10 μm/min.

Ion and fluid transport across surface epithelium

Under unstimulated conditions, the main form of transepithelial solute transport across human airway epithelia is active absorption of Na [17-19]. Active secretion of Cl can be induced transiently by several mediators [19]. Its magnitude,

however, is less than that of the baseline Na absorption. Under resting conditions, cultures of airway epithelium absorb fluid at ~5 $\mu l.cm^{-2}.h^{-1}$ [20], a rate which should reduce the depth of ASL at ~1 μm/min. Stimulation of Cl secretion by cAMP or $[Ca^{2+}]_i$ transiently reduces the rate of fluid absorption, but rarely results in net secretion of liquid [20].

Goblet cell discharge

Fluid may be drawn into the airway lumen by forces generated by the expansion of mucous granules released by goblet cells [21]. In healthy humans, goblet cells comprise ~1/6 of the columnar cells of the tracheobronchial epithelium [22]. Gravimetric analysis of tracings of published micrographs suggests that mucous granules occupy ~8% total cell volume or ~400 nl per cm^2 of airway surface. Making the conservative assumption that mucous granules swell 10-fold following discharge [21-23], then complete discharge of all goblet cell granules should increase the depth of ASL by ~40 μm.

ATP is the most potent inducer of goblet cell discharge [24, 25]. In the guinea-pig trachea, goblet cells are under autonomic control [26]. However, this is probably not the case in humans and most other mammalian species, in which goblet cells discharge only in response to local irritation [21, 27].

Osmotic and hydrostatic pressure

ASL has been variously reported as hypertonic [28] or hypotonic [29] with respect to plasma. Some of the confusion may arise from sampling inadequacies. In the most generally used approach, pieces of filter paper are applied to the airway surface, and withdrawn when saturated. As the paper used is generally much thicker than the depth of ASL, it damages the cells [30], and may sample liquid from the cells and from the interstitium beneath. The most recent estimates of ion content of human ASL suggest that it is hypoosmotic to plasma [29] thus favoring removal of liquid from the lumen.

The hydrostatic pressure of the airway interstitium is unknown. However, in most other tissues it is ~-2 mm Hg [31]. Again, this would favor movement of fluid from lumen to subepithelial space. However, in asthma, and other types of airway inflammation there is widespread extravasation in the airways [32], causing the submucosal tissue to become edematous. Subepithelial pressure is elevated during this extravasation as revealed by the marked dilation of the epithelium's lateral intercellular spaces [33]. In other epithelia, pressures of ~20 cm H_2O cause maximal dilation of the LIS [34] while substantially lower pressures markedly increase permeability to water and to solutes as large as proteins [35, 36]. In dog tracheal epithelium *in vitro*, pressures as low as 5 mm Hg increase permeability to water [P. Matsumoto and J.H. Widdicombe, unpublished], albumin and mannitol [37]. Should pressures of this magnitude exist during extravasation *in vivo*, then the resulting volume flows would account for the excess liquid and blood proteins in the airway lumen during inflammation [38]. Given the dramatic effect of subepithelial hydrostatic pressure on hydraulic conductivity [36], it is likely that

hydrostatic pressures generated by extravasation are the prime determinants of transepithelial volume flows during airway inflammation.

Surface tension

There are eight cilia per μm^2 of epithelial surface area [39]. Being 200 nm in diameter and 6 μm in length [39], they will increase the apical membrane surface area by ~30-fold. Surface area is also increased by the numerous microvilli between the cilia. It has been calculated that the forces of surface tension generated by the closely spaced cilia generate a pressure of ~2 atmospheres [7], corresponding to a concentration gradient of ~40 mOsM. This is probably greater than the bulk difference in concentration between ASL and interstitial fluid, and is almost certainly much greater than the local osmotic gradients generated by transepithelial salt transport [6]. In other words, fluid will tend to be held in place between the cilia, and as long as there are cilia there will be a periciliary sol.

Depth of ASL

Most microscopical methods are inadequate for assessing the depth of ASL. Previous techniques involving chemical fixation of the ASL are subject to several artifacts. Instillation of liquid fixative into the airway lumen may wash away the mucous gel [40, 41], and immersion fixation [3, 42] is open to the same criticism. Vascular perfusion maintains well the structural relationship between ASL and underlying tissues [41, 43]. However, during fixation by either vascular perfusion or by immersion, osmotic gradients will be set up which could alter the depth of ASL. Finally, marked shrinkage of the ASL may occur during the many steps involved in conventional tissue preparation.

Rapid freezing avoids the problems associated with chemical fixation. Frozen tissues, cryosectioned and then freeze-dried and substituted have provided excellent differentiation between the sol and the gel [2, 5]. However, freeze substitution is associated with tissue shrinkage of 11% in the linear dimension [44], and portions of the ASL may be fractured away during cryosectioning [45]. This approach is therefore of limited usefulness in determining quantitative changes in the depth of ASL.

Rapid freezing combined with low temperature scanning electron microscopy [46] is a technique used to examine cells and bulk tissue samples in the frozen hydrated state. This technique does not distort, substitute or dehydrate any tissue compartments, and it can resolve tissue compartments on the order of 1 nanometer. It therefore provides a snapshot in time of the fluid state of the tissue and can be utilized to determine depth of the ASL at various times during physiological stimulation.

After experimenting with various tissues, and several different ways of freezing and handling, we came up with the following approach for using LTSEM to visualize the ASL [47]. Cow tracheas were obtained from a nearby abattoir within five minutes of death, and transported to the laboratory at 4 °C in air saturated with water vapor. An epithelial sheet (~25 cm^2) with the submucosal glands intact was dissected away from the underlying cartilage and muscle, and cut into individual 1

cm^2 squares which were pinned out, mucosal side up, on sponges saturated with physiological saline, and maintained in a warm (37 °C) humidified atmosphere. Following a 30 min equilibration period, tissues were frozen when a cryoprobe with a flat polished copper surface, precooled to the temperature of liquid nitrogen (-196 °C) was rapidly brought into contact with the mucosal surface. Samples were maintained at <-180 °C, and processed as previously described for frozen pieces of lung [44]. First, probe tip and frozen tissue were rapidly covered with liquid nitrogen. The tissue was trimmed under liquid nitrogen with a high-speed circular dental saw, and mounted in a miniature vise built into an SEM stub. The specimen holder and tissue were transferred via an airlock to a cryochamber (Biochamber; AMRay, Bedford, MA) attached to the microscope which was maintained under vacuum (10^{-4} to 10^{-3} Pa) by a diffusion pump and at liquid nitrogen temperature by a cold shroud and a tank of liquid nitrogen. The specimen was moved within the chamber by a shuttle to an observation window. While viewed by a dissecting microscope, a precooled knife fractured the tissue at right angles to the epithelial surface. If necessary, fracturing was repeated until a clean, smooth fracture plane was obtained. Some samples were coated with gold (200 Å thickness) at this point, whereas others were radiant-etched for periods of up to 1 min prior to coating. To coat with gold, an electrode assembly was attached to the airlock, and the specimen stub was moved to a copper block immediately under the airlock. If coating was adequate as judged with the dissecting microscope, tissues were transferred from the cryochamber to an SEM stage cooled with a high pressure Joule-Thompson nitrogen refrigerator (< -180 °C). The stage was adjusted until the fracture surface was at right angles to the electron beam.

At high magnification in unetched samples, structural details of the tracheal epithelium, such as nuclei, cilia and mucous granules in goblet cells were evident, and the lining liquid was seen to be ~10 μm in depth (Fig. 2).

In unetched specimens, it was not possible to distinguish the periciliary sol from the mucous gel because both are probably more than 95% water. However, if the depth of the airway lining liquid was first increased by stimulating submucosal gland secretion with methacholine, then radiant-etching clearly revealed two layers (Fig. 3). The smaller size of the ice crystal voids in the layer furthest removed from the epithelium is consistent with a higher solute (mucin) content of this layer.

In control tissues, the combined depth of periciliary sol and mucous gel was 6.9 ± 0.2 μm. On stimulating gland secretion with methacholine, the depth increased to a maximum of 27.9 ± 1.3 μm after 2 min exposure. In the continued presence of methacholine, depth thereafter declined approximately linearly with time reaching 8.8 ± 0.4 μm after 30 min. We speculate that these changes in the depth of ASL represent transient gland secretion followed by fluid absorption across the surface epithelium. The increase in depth of 21 μm over 2 min corresponds to 63 $\mu l.cm^{-2}.h^{-1}$, in good agreement with our estimate of maximal gland secretion (in humans) of 60 $\mu l.cm^{-2}.h^{-1}$. The decrease in depth of 19.1 μm between 2 and 30 min corresponds to fluid absorption of 4.1 $\mu l.cm^{-2}.h^{-1}$, in good agreement with our measured values for fluid absorption across cultures of human tracheal epithelium [20] (under baseline conditions, the cow tracheal epithelium used in these studies was

predominantly Na absorbing [49], with ~80% of baseline short-circuit current inhibited by amiloride).

Thus, we have shown that LT-SEM can be used to view ASL, while avoiding the problems associated with older microscopic techniques. We found that sheets of bovine tracheal epithelium when rapidly frozen and viewed by LT-SEM maintained excellent preservation of epithelial ultrastructure. Radiant-etching distinguished two layers of ASL which we speculate correspond to the sol and gel. Because the current technique involves minimal changes in the dimensions of ASL during processing, it provides a useful approach to determining how changes in gland secretion, goblet cell discharge, and fluid transport by the surface epithelium affect the depth of ASL.

Composition of ASL

X-ray microanalysis is a technique used to determine elemental composition in numerous materials from biological tissue, to minerals and integrated circuits [50]. Energetic electrons in the beam of an electron microscope displace electrons from inner orbital shells of atoms of the sample. Electrons from outer orbitals drop into the vacated shells and X-rays of energies specific to each element are emitted. X-ray analyzers sort incoming X-rays (in counts per second) according to their energy level and generate a spectrum with Gaussian-like peaks for each element.

In preliminary studies we have examined freeze-dried Calu-3 cells, an airway cell line that forms tight junctions and shows active secretion of Cl [51]. When grown without adding medium to the mucosal surface ("air-interface feeding"), Calu-3 cells have a film of liquid on this surface ~15 μm in depth. After drying of the bulk tissue, we noticed that the ASL had shrunk but remained intact. Relatively thick sections (~50 μm) were taken of the samples. With an accelerating voltage of 200 keV, the electron beam penetrates a freeze-dried sample with only a small amount of spreading of the beam. Thus, the volume exposed to the electron beam is known, and reproducible spectra result.

Fig. 4 shows X-ray spectra of the ASL and epithelium for Calu-3 cells. The beam current was 6.5 mA which resulted in a 86 Å beam. The scanned area was 3 μm X 20 μm. Compared to the cells, the ASL shows relatively high sodium and Cl and very low potassium and phosphorus content. The Na and Cl signals are sufficiently strong to detect clear-cut differences in Na and Cl content between ASL and cytoplasm. We also note that the sulfur signal is well represented in the ASL spectrum, presumably reflecting the presence of sulfate on mucins [52]. Aluminum was used to coat the mucosal tissue surface, and provides a strong signal from the ASL.

We plan to use X-ray microanalysis in several types of study. First, we will measure S levels in the layers which we have tentatively identified as sol and gel on the basis of differences in size of ice crystal voids. If the layers do indeed correspond to sol and gel, the former should have a much lower S peak than the latter. Second, we will see how the elemental composition of ASL is changed by gland secretion, goblet cell discharge and ion transport across the surface epithelium. Third, we will

use human surgical specimens to test the conclusion of others [29] that Na and Cl levels of ASL are higher than normal in cystic fibrosis.

Figure Legends

Fig. 1. Sol and gel layers in cultured rabbit tracheal epithelium. Tissues were rapidly frozen, sectioned, freeze-dried and viewed by scanning electron microscopy. Note that the depth of the sol remains equal to the length of the cilia, though the thickness and consistency of the mucous gel (mu) varies. From Sanderson and Sleigh [2] with permission.

Fig. 2. Unetched epithelium. L–surface liquid, C–cilia, G–goblet cell. Horizontal field width=45 μm. From Wu et al. [47] with permission.

Fig. 3. Etched epithelium. The airway surface liquid can be differentiated into two layers on the basis of the size of the ice crystal voids. We have tentatively identified these layers as sol (S) and gel (G). Horizontal field width=115 μm. From Wu et al. [47] with permission.

Fig. 4. X-ray spectra from freeze-dried Calu-3 cells. The cell spectrum is the shaded area. The ASL is the unshaded region. The Al peak in the ASL spectrum comes from aluminum used to coat the mucosal surface of the specimen.

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