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Measurements of airway surface liquid height and mucus transport by fluorescence microscopy, and of ion composition by X-ray microanalysis

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Abstract

The respiratory tract is lined by a thin layer of fluid, the airway surface liquid (ASL), which plays a critical role in lung defense. The paper describes methods to determine the height of the ASL and corresponding mucus transport rates using fluorescent probes, and methods to determine the ionic composition of the ASL by X-ray microanalysis.

Keywords: Airway surface liquid; Fluorescent probes; Confocal microscopy; X-ray microanalysis; Cell culture; Animal models

1. Introduction

The respiratory tract is lined by a thin layer of airway surface liquid (ASL), which plays an important role in maintaining the sterility of the lung. This layer consists of two phases: mucus, which traps airborne particles and bacteria, and an underlying periciliary liquid layer (PCL), which lubricates the mucus and keeps it at an optimum distance from the cilia, enabling the mucus to be cleared from the airways either by ciliary beating or by cough [1]. The volume and composition of the ASL must be tightly regulated in order to maintain continuous mucus transport. However, defining the exact nature of the ASL has proved difficult. For example, published data on both the volume and composition of the ASL are very divergent and the ASL has been reported to vary from 7 to 70 μm in height [2,3] and from very hypotonic to hypertonic in concentration [4–9]. In addition to sampling from both humans and from animal models, airway epithelial cultures have also been investigated which similarly yielded a broad range of results with chloride (Cl^-) concentrations between 18 and 140 mM being reported [10–12]. A number of different techniques for sampling and analysis have been used, and it would appear that, possible species differences aside, one source of variation is the sampling technique.

This paper describes some of the current methods used to investigate the physiology of native ASL. Studies of ASL in the Chapel Hill laboratory are based on the use of vertical (XZ) laser scanning confocal microscopy to measure ASL height, as an index of ASL volume, coupled with epifluorescence measurements of mucus transport rates (Sections 2–6) [10]. In contrast, methods to determine the ion (elemental) composition of the ASL by X-ray microanalysis have been developed in the Uppsala laboratory (Sections 7–10).

2. Cell culture

A powerful aid to studying ASL physiology has been the development of suitable culture models that recapitulate in vivo airway epithelial morphology (Fig. 1). We seed primary airway cells directly onto 12-mm diameter inserts (Corning-Costar Transwell Collagen T-cols, Acton, MA, USA) following an overnight digestion of donor lungs in a protease solution [10], usually at a density of 2.5×10^5

Abbreviations: ASL, airway surface liquid; CCD, charge-coupled device; MCC, mucociliary clearance; PCL, periciliary liquid layer; PFC, Perfluorocarbon; PBS, phosphate-buffered saline; SEM, scanning electron microscope.

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cells per cm^2. Cultures usually become confluent within 3–5 days and are then grown with an air–liquid interface, which means that culture medium is omitted from the mucosal surface post-confluence allowing production of native ASL. These conditions are thought to trigger the cultures to fully differentiate, and over a period of about 2 weeks they form a layer of ciliated cells (70%) interspersed with goblet cells (30%) over layers of undifferentiated basal cells (Fig. 1). Overlying the mucosal—most cells is the ASL, which resembles ASL in vivo since it is delineated into PCL and mucus (Fig. 1).

Like proximal airways in vivo [13], our airway cultures are predominantly Na^+/volume absorbing, and hence, absorb excess ASL volume with time [10,14]. To standardize the starting ASL height/volume, cultures are prewashed to remove excess ASL/mucus and the desired fluorescent probes are added either with the final wash (after which liquid is aspirated to minimal levels by a vacuum pipette) or in a bolus of liquid (i.e., 20–50 μl) that is left on the cultures for extended periods (i.e., more than 2 days). Cultures are then left in a 100% humidified incubator between readings [10]).

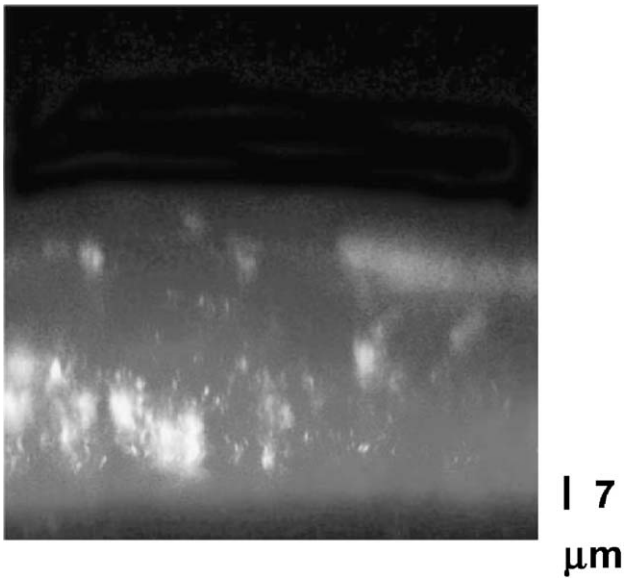

Fig. 2. Imaging of ASL by XZ confocal microscopy. Typical image of ASL (gray band) and fluorescent microspheres (light gray particles), which discontinually associate with mucus. Note that the microsphere-free region at the bottom of the image is the PCL. The scale bar is 7 μm.

3. Choice of probes

We typically label ASL with Texas Red conjugated to 10 kDa dextran, which dissolves in the ASL and is relatively impermeable across the epithelium (Fig. 2). However, Oregon green-dextran has also been used and serves the same purpose [10]. Mucus is labeled with 100 nm fluorescent microspheres, which co-localize to "strands" in the mucus layer (Fig. 2). Larger microspheres of another colour (i.e., blue fluorescence) can also be added at the same time to track mucus transport rates (see Section 5). We have recently used BCECF- and SNARF-dextrans to measure ASL height and pH simultaneously [15], and no doubt other probes will prove useful in the future. An extensive list of fluorescent dyes can be found in the Molecular Probes catalogue (http://www.probes.com).

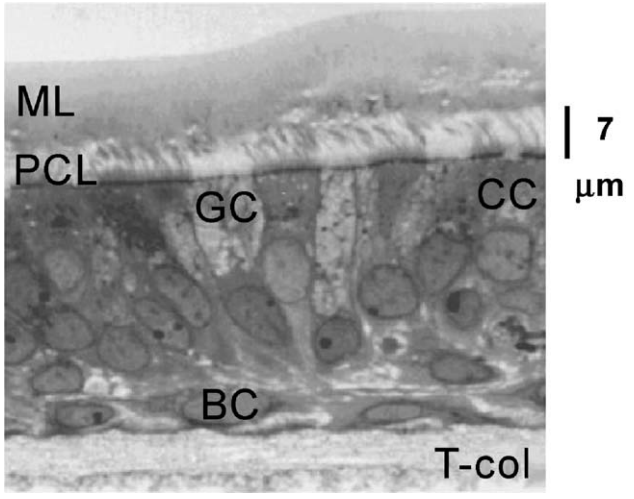

Fig. 1. Differentiated airway epithelial cultures. Human tracheo-bronchial epithelia are grown at an air–liquid interface on semi-permeable supports (T-cols) for 2–5 weeks after seeding. ML, mucus layer. PCL, periciliary liquid layer. GC, goblet cell. CC, ciliated cell. BC, basal cell. T-Col, semi-permeable culture insert. Scale bar is 7 μm.

4. Use of perfluorocarbons

Perfluorocarbons[1] (PFCs) are added to the mucosal surface during ASL measurements to prevent evaporation [14,16], after which, cultures are returned to the 100% humidified incubator. Advantage is taken of the fact that FC-72 PFC has a relatively low boiling point (56 °C) compared to FC-77 PFC, which has a much higher boiling point of 97 °C. Thus, the shorter-lasting FC-72 is typically applied before addition of any compounds (to measure initial baseline heights) whilst FC-77 is added after addition of the compound to prevent ASL evaporation. Since addition of compounds (e.g., amiloride, ATP) to the ASL in a liquid vehicle would result in a direct increase in ASL volume, compounds are also added as dry powders suspended in PFC (FC-72; 3M Company, Minneapolis, MN, USA). We have also used jet milling to produce ultra-fine particles, which distribute very well in the PFC, although these particles become hygroscopic.

[1] Note that the use of PFCs may be prohibited in some countries.

5. XZ confocal microscopy

We use inverted microscopes with high numerical aperture (1.2 NA) water immersion lenses to image from below the cultures. Oil lenses can also be used, but they have a smaller working distance than water lenses, which can sometimes make obtaining a suitable image difficult. Lower NA dipping or dry lenses can also be used (e.g., 0.7 NA), but we have found them to give poorer resolution whilst XZ-scanning. Cultures are placed on a coverslip mounted in a chamber, which can be either heated or not. Several commercial designs are available including heated chambers from World Precision Instruments and unheated chambers from Molecular Probes (Eugene, OR, USA). Upright microscopes can also be used but they require either dipping or dry lenses, which typically yield less accurate XZ scans and also require the use of larger cultures (24-mm diameter minimum insert size), which may become an issue if the number of cells available for culturing is limited.

6. Measurement of mucus transport rates

Our well-differentiated (ciliated) bronchial cultures coordinate ciliary beating to spontaneously transport mucus across the mucosal surface. To measure rates of mucus transport, our laboratory has employed fluorescent microspheres (1 μm, Molecular Probes), which when added to the cultures, partition into the mucus layer. The microspheres are then imaged in real-time using a conventional inverted epifluorescence microscope with a 5 × dry lens attached to a charge-coupled device (CCD) camera (either colour or black and white). The rate of mucus transport is determined from 5-s exposure images (Fig. 3). Here, the movement of the single microsphere produces a streak of fluorescence in the image, with the length of the streak corresponding to the distance the microsphere has been transported in 5 s. Alternatively, the rates of mucociliary clearance (MCC) can be determined from measurements of microsphere location changes from sequential images utilizing a high-speed CCD camera. The later technique is useful for constructing videos of MCC (see Ref. [17]).

This technique has revealed that the entire mucus layer moves as a single network as evidenced by the increase in linear velocity versus the distance from the apparent center of rotation (Fig. 3b; Ref. [10]). Consequently, the angular velocity of mucus transport can be determined in cultures that exhibit radial transport of microspheres.

7. Preparation and analysis of frozen hydrated airways from experimental animals

The trachea or bronchi are removed from deeply anesthetized animals and dissected in a specially designed humidified chamber. Dissected tissue pieces are then frozen in liquid propane cooled by liquid nitrogen. For analysis, the tissue pieces are placed with the mucosal side either up or pointing sideways onto a specimen holder and transferred (at liquid nitrogen temperature) to a scanning electron microscope (SEM) equipped with a cold stage, which maintains the specimen at − 190 °C throughout the analysis. The samples are coated with a thin carbon layer on the cold stage and an accelerating voltage of 9–10 kV is applied. A total of 8–10 analyses per sample are carried out with an energy-dispersive spectrometer system for 500 s with a beam size of 200 nm. For quantitative analysis, the data are compared to the results obtained on a drop of frozen salt solution of known composition and analyzed using the ratio of characteristic to continuum intensity [18].

8. Collection of tracheal fluid on ion-exchange beads

Ion exchange beads (Sephadex G-25; 20–40 μm diameter; Pharmacia, Uppsala, Sweden) are spread evenly on the surface of the dissected pieces of the pig trachea and left for 10–30 min in the humidified chamber (described above;

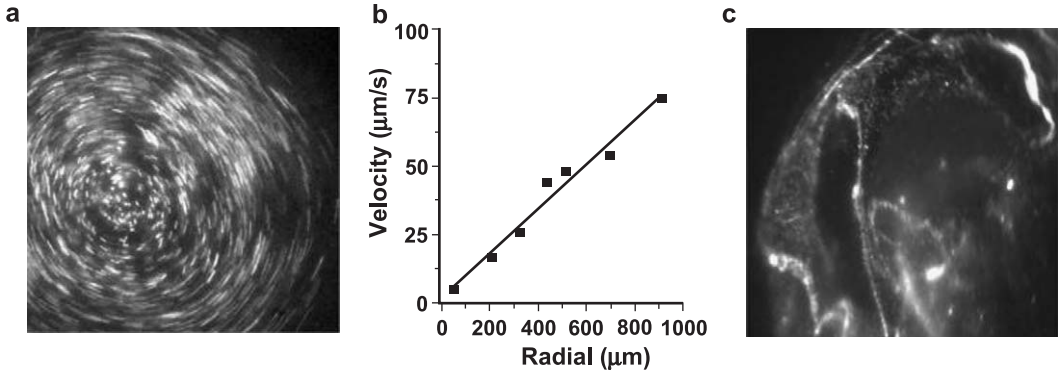

Fig. 3. Epifluorescence microscopy of rotational mucus transport by airway epithelia. (a) Five-second exposure photograph of fluorescent microspheres bound to mucus on a normal airway culture. (b) Plot of microsphere velocity against distance from the center of rotation taken from (a). The slope may be used to normalize transport to a set distance from the center (e.g., 1 mm). (c) Five-second exposure image of a stationary mucus plaque on a CF culture after excess liquid has been absorbed by Na^+-led hyperabsorption [10,16].

shorter times for smaller animals). Sephadex is the trade name for a cross-linked dextran gel with ion-exchange capacity, and these beads saturate with salt solution within about 5 min [19]. The beads may be sprayed on to a sample via a syringe, or mounted on a filter paper (see Section 9) and it is important that the beads are well spread and do not stick together. After absorption of the ASL, the beads are recovered by flushing with hydrophobic, volatile silicone oil and collected in a watch glass [20]. Under a preparation microscope, all adhering fluid and debris is removed from the beads, and single beads are transferred onto nylon specimen grids, which are submerged into the oil. It is important that adhering mucus is removed from the beads since this may alter readings for some elements. The grid and beads are slowly lifted out of the oil-bath and mounted onto an aluminum specimen holder covered with round carbon adhesive tape and left at room temperature until the oil evaporates. The samples are then carbon coated. X-ray microanalysis of the beads is performed with the instrumentation described above at 20 kV for 100 s with a 100 nm beam. Typically 10–12 beads are analyzed from each sample (one measurement per bead). For quantitative analysis, the data are compared to the results obtained on beads soaked in salt solutions of different concentrations (50–250 mM), or with beads soaked in serum or plasma from the same animals and analyzed chemically.

9. Collection and analysis of human nasal fluid

Sephadex G-25 beads are applied to double-sided tape (3M Company) attached to filter paper (2×2 mm^2; Whatman, Springfield Mill, UK) and inserted into the subject's nostril for 10–20 min (longer time is needed for cystic fibrosis (CF) patients because of the viscosity of the nasal secretions). After this period, the beads are placed under a preparation microscope and carefully washed in hydrophobic volatile silicon oil to ensure that no fluid remains outside the beads. The beads are then separated and individually moved to a nylon electron microscopy grid, prepared and analyzed as described above.

10. Collection and analysis of liquid from airway epithelial cultures

10.1. Liquid–liquid interface cultures

Airway epithelial cell lines are grown on membrane inserts in a 24-well chamber in a conventional liquid–liquid interface culture system for 4–7 days. Confluency of the culture is checked by measurement of the transepithelial resistance ($\geq 400\Omega.cm^2$). Cultures are then covered with a layer of phosphate-buffered saline (PBS) for 24 h. The entire culture and insert, including the covering liquid layer, is then frozen in liquid propane cooled by liquid nitrogen, transferred in the frozen state to a SEM equipped with a cold stage and analyzed as described in Section 8. Alternatively, Sephadex G-25 beads are placed on the cell culture for 20 min and the membrane insert cut from the holder, placed in a watch glass and covered with silicon oil. Further preparation and analysis of the beads is as described in Section 9.

10.2. Air–liquid interface cultures

The cells are first grown as liquid–liquid interface culture; then the culture medium is removed from the apical surface and cells are cultured for 1 month [10] (see also Section 2); during this period the apical surface is washed with PBS twice a week, during which time ciliogenesis occurs. Sephadex-G25 beads are added to the ASL, left to equilibrate for 20 min and then transferred to grids and analyzed as described above.

11. Conclusions

In conclusion, we have described direct measurements of ASL volume and composition by confocal microscopy and X-ray microanalysis, respectively. These techniques may be correlated to bioelectric and mucus transport measurements to better understand integrated airway physiology. We believe that these methods will help pave the way towards a better understanding of respiratory diseases characterized by altered ASL rheology such as asthma, CF and chronic pulmonary obstructive disease.

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