Fluid transport across cultured rat alveolar epithelial cells: a novel in vitro system

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Fang, Xiaohui, Yuanlin Song, Rachel Zemans, Jan Hirsch, and Michael A. Matthay. Fluid transport across cultured rat alveolar epithelial cells: a novel in vitro system. Am J Physiol Lung Cell Mol Physiol 287: L104–L110, 2004. First published February 27, 2004; 10.1152/ajplung.00176.2003.—Previous studies have used fluid-instilled lungs to measure net alveolar fluid transport in intact animal and human lungs. However, intact lung studies have two limitations: the contribution of different distal lung epithelial cells cannot be studied separately, and the surface area for fluid absorption can only be approximated. Therefore, we developed a method to measure net vectorial fluid transport in cultured rat alveolar type II cells using an air-liquid interface. The cells were seeded on 0.4-μm microporous inserts in a Transwell system. At 96 h, the transmembrane electrical resistance reached a peak level (1,530 ± 115 Ω·cm²) with morphological evidence of tight junctions. We measured net fluid transport by placing 150 μl of culture medium containing 0.5 μCi of 131I-albumin on the apical side of the polarized cells. Protein permeability across the cell monolayer, as measured by labeled albumin, was 1.17 ± 0.34% over 24 h. The change in concentration of 131I-albumin in the apical fluid was used to determine the net fluid transported across the monolayer over 12 and 24 h. The net basal fluid transport was 0.84 ± 0.25 μl·cm⁻²·h⁻¹. CAMP stimulation with forskolin and IBMX increased fluid transport by 96%. Amloride inhibited both the basal and stimulated fluid transport. Ouabain inhibited basal fluid transport by 93%. The cultured cells retained alveolar type II-like features based on morphologic studies, including ultrastructural imaging. In conclusion, this novel in vitro system can be used to measure net vectorial fluid transport across cultured, polarized alveolar epithelial cells.

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Isolation and culture of rat alveolar type II cells. Alveolar epithelial type II cells were isolated from pathogen-free male Sprague-Dawley rats by enzymatic digestion and panning on plates coated with IgG to remove macrophages and leukocytes, as previously described (10, 16). Cell viability was >95% by trypan blue exclusion, and the yield was 15–25 × 10⁶ cells per rat. Cell purity assessed by Papanicolaou staining was >90%. Preparations containing <90% type II cells were discarded. The cells were prepared in culture medium DME H-21 containing 10% FBS (University of California-San Francisco cell culture facility) and were seeded at a density of 1.6 × 10⁶ cells/cm² in tissue culture-treated polycarbonate Transwell membranes with 0.4-μm pores and a surface area of 0.33 cm² (Corning, NY) (Fig. 1). The culture medium containing 10% FBS was added to the lower compartment of the Transwell. The cells were maintained in a 37°C 5% CO₂ incubator. After 48 h, the cells formed a confluent monolayer. The monolayer was washed with medium to remove unattached cells. After the confluent monolayer was achieved at 48 h, transmembrane electrical resistance was measured daily with an Ag-AgCl electrode. The electrode was sterilized with 70% alcohol for 10 min, rinsed twice with PBS, and placed in the DME H-21 for 1 h to reach electrical balance before measurement. Measurements were done before the culture medium was changed. After 120 h of growth, the cells in selected Transwells were counted. Cells were first washed twice with PBS solution, and then 0.1 ml and 0.5 ml of 0.25% trypsin were added to the upper and lower compartments of the Transwell, respectively. The Transwell was incubated at 37°C for 15–20 min, and the detached cells were collected and counted.

Rat alveolar epithelial monolayer permeability to protein. To determine whether the cultured monolayer was mostly impermeable to protein, we measured the protein flux across the rat alveolar epithelial cells by measuring the unidirectional flux of labeled 131I-albumin from the apical (upper compartment) to the basolateral side (lower compartment). The cells formed confluent monolayers 48 h after seeding, and the culture medium in both the upper and lower compartments of the Transwell was changed. At 72 h, the fluid in the upper compartment was removed with gentle aspiration, and the cells were then grown in an air-liquid interface. At 96 h, 150 μl of serum-free medium containing 0.5 μCi/ml 131I-albumin were pipetted

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on the top of the cells in a humidified tent, and the plate was returned to the 37°C 5% CO₂ incubator. Twenty-four hours after 131I-albumin was pipetted in, all fluid in the upper and lower compartments of the Transwells was aspirated, and the total 131I was measured in the gamma counter (Packard MINAXI 5000 series). Trichloroacetic acid (20%) was added to the upper and lower compartments of selected Transwells to determine 131I binding to albumin as we have done before (33). There was <1.5% of free 131I.

Paracellular permeability of rat alveolar epithelial cell monolayer. [14C]mannitol was used to measure the paracellular permeability of the rat alveolar epithelial monolayer (n = 24 in four different preparations). Fluid containing 0.05 μCi of [14C]mannitol was pipetted into the upper compartment of the Transwell. The plate was incubated at 37°C. Samples were aspirated from both upper and lower compartments at 5 min, 12 h, and 24 h and counted in an LS 6500 Multipurpose scintillation counter (Beckman Coulter) for 40 min. [14C]mannitol permeability was expressed as μmol·cm⁻²·h⁻¹ and percent change over 12 and 24 h.

Measurement of fluid transport across the rat alveolar epithelial monolayer with an isotope-labeled albumin method. To validate this method of measurement for the net fluid transport across a cell monolayer, we measured the 131I-albumin concentration change over time in empty Transwell membranes (no cells) that were sealed with paraffin to simulate an impermeable barrier. In this manner, we estimated the magnitude of 131I-albumin adsorption to the filter and the plastic walls of the Transwell, as well as the degree of evaporation, both of which are potential confounding factors in the estimation of fluid transport by this method.

Fluid in the upper and lower compartments of the Transwell was maintained at the same level to avoid a hydrostatic pressure gradient. 131I-albumin was used as a volume marker, as in our previous in vivo studies. The measurement of fluid transport from the apical to basolateral membranes of the epithelial cell monolayer was done 96 h after the isolation and plating of the cells, 24 h after an air-liquid interface had been achieved. Medium (150 μl) containing 0.5 μCi 131I-albumin was pipetted into the apical chamber of the Transwell in the humidified tent. Five minutes after the 131I-albumin was added, 20 μl of the medium were aspirated as the initial sample. The 5-min period was allowed for adherence of protein to the apical membrane and potential initial dilution. The plate was then incubated for 12–24 h in the 37°C 5% CO₂ incubator with 100% humidity. After either 12 or 24 h, 20 μl were aspirated from the upper compartment of the Transwell as the final sample. The samples were weighed and counted in the gamma counter. Fluid absorption was calculated as in our prior in vivo studies (31, 32, 41): Fluid absorption = [1 – (radioactivity in the initial sample/weight of initial sample)/(radioactivity in the final sample/weight of final sample)] × 100%. Forskolin and IBMX (10⁻⁵ M each, n = 36 wells) or amiloride (10⁻⁵ M, n = 24 wells) was added to the apical culture medium in selected studies. Ouabain (10⁻⁵ M, n = 12 wells) was added to the culture medium in the lower compartment in other studies.

We also measured the change in permeameter marker concentration in the lower compartment of selected Transwells to calculate fluid movement from the basolateral to apical surface to ensure that the calculated fluid transport is equal and opposite in the two directions. For measurement of fluid dilution in the lower compartment, 600 μl of medium containing 131I-albumin were pipetted into the lower compartment, and 150 μl of medium were pipetted into the upper compartment. Approximately 5 min after the 131I-albumin was added, 20 μl were aspirated from the lower compartment as the initial sample. After 24 h of incubation in the 37°C 5% CO₂ incubator with 100% humidity, another 20 μl were aspirated from the lower compartment of the Transwell as the final sample. Fluid movement from the basolateral to apical side was calculated by the same method described in Measurement of fluid transport across the rat alveolar epithelial monolayer with an isotope-labeled albumin method (n = 18 in each group).

Cell markers for alveolar type II and alveolar type I phenotypes. Through the use of cell surface markers of alveolar type I (ATI) and type II (ATII) cells, other investigators have found that ~95% of the cells cultured as in these studies have an ATII phenotype (20, 32), with little conversion to an ATI phenotype in the first 96 h of culture. We addressed this issue in our culture conditions. Cells were seeded on 0.33-cm² clear Transwell membranes for 48–120 h (with an air-liquid interface), and then cells were preincubated at 37°C in DMEM with Lysotrack green DND-26 (150 μmol/l, 30 min; Molecular Probes), which is a fluorescent dye to selectively stain lamellar bodies in primary culture ATI cells (1, 18, 19). Images were obtained with a Nikon inverted microscope (TE 2000-E) and Simple PCI Advance Image Capture system. Cells were also trypsinized from the membrane and stained with aquaporin-5 (AQ5P) antibodies (Chemicon) to determine the relative numbers of ATI-like cells.

Ultrastructure of cultured rat ATII cells. Freshly isolated rat ATI cells were seeded in Transwell membranes at a density of 1.0×10⁵/μl. Then the cells were cultured under the same conditions as described in Isolation and culture of rat alveolar type II cells. At 120 h, the monolayers were fixed in 3% (wt/vol) Karnovsky fixative for 1 h at 0°C. In addition, cells on Transwell membranes were scraped off with a rubber policeman, and cell suspension was centrifuged briefly at 160 g. The resulting cell pellet was then fixed in 3% Karnovsky fixative for 1 h at 0°C. Both the cells from monolayer membranes and the pellet were postfixed 1 h in 1% paraformaldehyde aqueous uranyl acetate and Reynolds lead citrate, then sections were screened with a JEOL 1200 EX transmission electron microscope operating at 80 kV.
again washed four times with PBS/FSG/SAP/RNase for 10 min and then were incubated with a mixture of secondary rhodamine-labeled anti rabbit-IgG (Sigma) and 4',6-diamidino-2-phenylindole dihydrochloride (Molecular Probes) for 45 min. The slides were mounted with Vectashield mounting medium and covered with coverslips. Images were obtained by a Bio-Rad MRC-1024 laser scanning confocal microscopy system.

Statistical analysis. Results are expressed as the means ± SE of values from at least three separate rat experiments, each done with six wells. Comparisons between two groups were made by an unpaired, two-tailed t-test. Comparisons between more than two groups were made by a one-way analysis of variance and with Tukey’s post hoc test. P < 0.05 was taken as statistically significant.

RESULTS

Transmembrane electrical resistance and phenotype of cultured ATII cells. Approximately 48 h after plating, the cells developed measurable transmembrane electrical resistance, which peaked at 96 h (Fig. 2A). Because daily measurements may alter the cells and underestimate the transmembrane electrical resistance, we also measured the resistance only at 120 h in some Transwells (n = 24). In these Transwells, the peak transmembrane electrical resistance reached 1,530 ± 115 Ω·cm². Tight junctions were also evident by visualization of ZO-1 protein in the cultured monolayers 120 h after plating (Fig. 2B). Therefore, the 24-h period between 96 and 120 h after initial plating of the cells was selected for the fluid transport experiments.

To define the phenotype of cultured ATII cells, cells were grown on permeable Transwell membrane (with an air-liquid interface). Cells were trypsinized from the membrane and stained with AQP5 antibody. Less than 5% of the cells stained positive for AQP5 during the time (96–120 h) in which the fluid transport studies were done. At both 48 h and 120 h, the monolayer showed that the cells had lamellar bodies (Fig. 3, A and B). There was no difference between the numbers of ATII cells from 48–120 h for cells grown on Transwell membrane. Electron microscopy of the cells showed that they contained lamellar bodies and microvilli (Fig. 3C).

Protein and mannitol flux across the rat alveolar epithelial type II cell monolayer. Flux of the bound 131I-albumin across the cell monolayer from the apical to the basolateral surface was 1.17 ± 0.34% over 24 h. Transwells with protein permeability >3% were discarded. Approximately 5% of the monolayers were not used for this reason. [14C]mannitol flux measured across the alveolar epithelial monolayer at 12 and 24 h was 8.1 × 10⁻⁶ and 14.4 × 10⁻⁶ mol·cm⁻²·h⁻¹ (5.4% over 12 h and 9.6% over 24 h), respectively (Fig. 4).

Fluid transport across cultured rat alveolar epithelial type II cells. Measurement of the 131I-albumin concentration change
Measurement of Fluid Transport with New In Vitro System

**DISCUSSION**

Our present understanding of active ion transport, barrier resistance, and fluid transport across the alveolar epithelium has been derived from both in vivo and in vitro studies. Insights into the specific mechanisms of alveolar epithelial cell transport have often derived from in vitro experimental design because there is more precise control of experimental variables. One of the earliest studies of the isolated alveolar epithelium used amphibian lung (6, 15). A few years later, rat ATII cells were harvested and plated on both porous and nonporous substrata (17, 28). On plastic culture dishes, numerous domes were formed, indicating that the cells exhibited active transport and formed occluding junctions. Amiloride and ouabain inhibited dome formation, whereas terbutaline stimulated dome formation, indicating vectorial sodium-dependent fluid transport from the apical to the basolateral surface. More detailed information regarding ion transport across ATII cells was obtained by culturing alveolar epithelial cells on porous supports, mounting them in Ussing chambers, and measuring $I_{sc}$ and ion flux under voltage-clamp conditions (3, 23, 24, 26, 31, 42). The $I_{sc}$ in an ATII cell monolayer corresponds closely to net sodium absorption. However, these methods cannot provide a quantitative measurement of net fluid transport.

Fluid absorption can be detected by making use of capacitance measurement (46). A double-sided capacitance microprobe technique can measure very small quantities of fluid transport in airway epithelia (21, 45). However, this technique is complicated, and removing probes while changing to fresh bathing solution in the chamber might alter the experimental results. Therefore, on the basis of previous experiments, we developed an improved method to quantify net fluid transport across cultured ATII cells by measuring the change in concentration of a nearly impermeant marker over time.

To minimize evaporative losses, the studies were done in a tent in which 5% CO$_2$ water vapor at 37°C was continuously circulated to maintain humidity at 90–100%. This procedure should have the same effect as coating the apical solution with mineral oil (43). The monolayer was relatively protein impermeable, since $^{131}$I-albumin flux was only 1.17 ± 0.34% over 24 h. Paracellular permeability as measured by $[^{14}$C]mannitol transport was only 14.4 × 10$^{-6}$ μmol·cm$^{-2}$·h$^{-1}$ over 24 h. Tight junctions formed by the alveolar epithelial type II cell monolayer at 120 h were also demonstrated by the staining for the ZO-1 protein in cell monolayers grown on glass slides. It was not technically feasible to do the ZO-1 imaging studies on the Transwell filter. The impermeability of the monolayer to protein validates this macromolecular approach to measuring net fluid transport, as in our in vivo studies (31, 32).

Fig. 4. $[^{14}$C]mannitol flux across the rat alveolar epithelial monolayer over 12 and 24 h. The y-axis is the $[^{14}$C]mannitol flux in 10$^{-6}$ μmol·cm$^{-2}$·h$^{-1}$. Experiments were done in 4 different cell preparations ($n = 24$ in each time point). *$P < 0.05$, data as means ± SE.

Fig. 5. Comparison of the rates of fluid movement in opposite direction in the Transwells. Experiments were carried out under basal and forskolin + IBMX-stimulated conditions. Experiments were done in 4 different preparations ($n = 18$ in each group). The y-axis is fluid movement in μmol·cm$^{-2}$·h$^{-1}$. *$P < 0.05$ compared with basal, data as means ± SE.
The transmembrane electrical resistance across the cultured monolayers rapidly increased after 48 h. The peak resistance level was at 96 h and was maintained for another 48–72 h. Therefore, we selected the 24-h period between 96 and 120 h after the plating of cells as the experimental time frame for the fluid transport studies. The relationship between the volume of transported fluid and time was linear, suggesting a constant rate of transport. These data provide evidence that this in vitro system is suitable for measuring net fluid transport in rat alveolar epithelial cell monolayers.

Basal fluid transport in rat ATII cells was 0.84 μl·cm⁻²·h⁻¹. cAMP stimulation increased fluid transport by ~96% over baseline. The inhibition of cAMP-stimulated fluid transport by amiloride suggests that functional epithelial sodium channels (ENaC) were present (20, 31). As expected, ouabain inhibited ~90% of fluid transport, which is consistent with the results of our previous studies in the in situ sheep lung and human lungs (39, 40).

An issue common to any study of cultured alveolar epithelial cells is the definition of the phenotype, since there is ample evidence that ATII cells gradually transition to an ATI-like phenotype after several days in culture (12). Early experiments established that ATII cells plated on plastic culture dishes rapidly lose characteristics that are associated with a differentiated type II cell phenotype (9, 11). Most surfactant protein expression is downregulated (25), and some transport proteins, such as Na-phosphate, and Na⁺-K⁺-2Cl⁻ cotransport vanish (4, 5). After 7 days in culture, rat ATI cells demonstrate decreased expression of the α₁-subunit and increased expression of the α₂-subunit of Na⁺-K⁺-ATPase, as shown by both mRNA and protein levels (37). In addition, after several days in culture, ATII cells demonstrate decreased expression of surfactant protein C. As they lose their ATII characteristics, cultured alveolar cells develop an ATI-like phenotype, as shown by increased expression of AQP5 and α₂-Na⁺-K⁺-ATPase after 7 days in culture (36). The precise definition of the phenotype of cultured alveolar epithelial cells is particularly important in the setting of the ongoing uncertainty about the relative contributions of ATI and ATII cells to fluid transport. Although considerable evidence has indicated that the ATII cell plays the principal role in edema clearance, recent data have suggested that the ATI cell may also contribute significantly to clearance. Freshly isolated ATI cells contain all three subunits of ENaC, as well as the α₁- and β₁-subunits of Na⁺-K⁺-ATPase, suggesting that ATI cells possess the machinery to contribute significantly to the maintenance of alveolar fluid balance (22, 36).

In the experiments presented here, the cells were studied between 96 and 120 h, a time period when the cells are known to have features of both types I and II cells (6, 31). However, recent work indicates that growth of cultured cells in an air-liquid interface preserves the ATI phenotype, including the expression of highly selective sodium channels (20). In this study, the cells were grown in an air-liquid interface for 24 h before the transport studies. Immunostaining of the cell monolayer growing at 120 h with Lysotracker green DND-26 showed that most of the cells still had lamellar bodies, a typical characteristic of ATII cells. Electron microscopy also confirmed that the cells studied here maintained an ATII phenotype. Therefore, we conclude that the rates of fluid transport measured probably reflect primarily the transport properties of ATII cells.

One of the advantages of our new in vitro system is that the surface area is controlled and we can study one cell type, in this case type II cells. In our in vitro studies, the rate of transport by the cultured ATII cells was 0.84 μl·cm⁻²·h⁻¹. How does this rate relate to the anticipated in vivo transport properties of type II cells in the intact rat lung? Interestingly, on the basis of several studies we have done in anesthetized, ventilated rats (31), the net basal fluid clearance rate was 27.4 ± 6.6% (mean ± SD) over 1 h in rat lungs (instillate in both lungs). In these studies, ~75% of the alveolar surface has been covered with the instillate. The total basal, unstimulated transport rate estimated in the in vivo setting is 0.18 μl·cm⁻²·h⁻¹, which was lower than in these in vitro studies. This calculation suggests that the in vivo fluid transport rates in these studies are not low. In addition, this calculation provides some interesting additional information that may have value in estimating the relative contribution of type II and type I cells in the intact lung. If the basal in vitro transport rate for the type II cells in this study approximates their in vivo capacity, then type II cells
transport at a rate that is four- to fivefold faster than would be expected by their surface area alone. This is certainly a tenable possibility, since type II cells are metabolically very active and have more easily detectable concentrations of sodium transport proteins than the nearby type I cells. However, this calculation also indicates that type II cells probably do not account for all of the alveolar fluid transport. If we assume that the type II cells occupy 3.7% of the rat lung surface area as previously published (7), then type II cells could account for 17% of basal fluid transport in the intact rat lung. There would still be 83% of fluid transport to be accounted for, which could be generated by type I cells and perhaps distal airway epithelial cells. By this calculation the type I cells, which occupy 96.3% of the alveolar surface, would account for most of the remaining fluid transport, which would be at a lower rate per surface area than type II cells. Therefore, these data support the hypothesis, based on other recent data (2, 22, 36, 37), that type I cells may contribute to fluid transport, although the contribution per cell based on surface area would be less than that of the type II cell. In summary, these studies demonstrate that an in vitro system can be adapted to provide a useful new method for the measurement of net vectorial fluid transport across alveolar epithelial cell monolayers. Although we used radioactively labeled albumin as the impermeant indicator, it should also be possible to use a fluorescently labeled protein in this model. The system should also be useful for studies of drug absorption and protein transport by isolated alveolar and distal airway cells.

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REFERENCES


