X-ray Microanalysis of Apical Fluid in Cystic Fibrosis Airway Epithelial Cell Lines

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Key Words

Ion transport • Airway surface liquid • Cell culture • Cystic fibrosis • pH measurements • X-ray microanalysis

Abstract

The ionic composition of the fluid lining the airways (airway surface liquid, ASL) in healthy subjects and patients with cystic fibrosis (CF) has been a matter of controversy. It has been attempted to resolve conflicting theories by using cell cultures, but published results show a wide variety of values for the ionic concentrations in the apical fluid in these cultures. To investigate CFTR-mediated HCO_3^- conductance and the role of HCO_3^- in regulating ASL pH we determined the pH of the fluid covering the apical surface of airway epithelial cells. A normal (16HBE14o-) and a CF (CFBE41o-) bronchial epithelial cell line were grown on membrane inserts in both a liquid-liquid interface culture system for 7 days, and in an air-liquid interface culture system for one month. The elemental composition of the fluid covering the apical surface was determined by X-ray microanalysis of frozen-hydrated specimens, or by

X-ray microanalysis of Sephadex beads that had been equilibrated with the apical fluid. Analysis showed that the apical fluid had a Na^+ and Cl^- concentration of about 80-100 mM and thus was slightly hypotonic. The ionic concentrations were somewhat higher in air-liquid interface than in liquid-liquid interface cultures. The apical fluid in CF cells had significantly higher concentrations of Na and Cl than that in control cultures. In control cultures, the concentrations of Na and Cl in the apical fluid increased if glibenclamide, an inhibitor of the cystic fibrosis transmembrane conductance regulator (CFTR) was added to the apical medium. Exposing the cells to the metabolic inhibitor NaCN also resulted in a significant increase of the Na and Cl concentrations in the apical fluid. The results agree with the notion that these cell cultures are mainly absorptive cells, and that ion absorption by the CF cells is reduced compared to that in normal cells. The pH measurements of the fluid covering the apical part of cell cultures support the notion that bicarbonate ions may be transported by CFTR, and that this can be inhibited by specific CFTR inhibitors.

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Introduction

The congenital, hereditary disease cystic fibrosis (CF) is caused by a mutation in a gene coding for a cAMP-regulated chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR). Despite the fact that the genetic defect has been known since 1989 [1], there is still disagreement on how the defect in CFTR leads to the various clinical symptoms of the disease, the most serious of these being chronically obstructive airway disease. Several theories have been put forward to describe the link between the defective CFTR and airway disease (reviewed in [2]). The most prominent among these theories are the "low salt hypothesis" and the "low volume hypothesis". In summary, the "low salt hypothesis" claims that the fluid covering the airway epithelium, the airway surface liquid (ASL) normally is hypotonic, but that its salt content in CF patients is higher due to deficient absorption of Cl^- ions by the airway epithelial cells, which would lead to a reduced activity of defensins, and a greater susceptibility to infections [3, 4]. The "low volume hypothesis" on the other hand centers on the fact that the defect in CFTR causes increased Na^+ absorption in CF epithelia by the epithelial Na^+ channel (ENaC). This would result in increased water absorption, an elevated Cl^- concentration in the ASL and a decreased volume of water-deficient viscous mucus [5-7]. Knowledge of the exact Cl^- concentration would help to solve the controversy. It has been attempted to resolve the conflict by measuring the ionic composition of the ASL but a recent review by Verkman et al. [2] shows that published values for the Na and Cl content of normal ASL (in various species including human, and using different sampling and analysis techniques) vary between less than 10 mM and around 140 mM. No agreement has been reached in the literature on whether the ASL in CF has an ionic composition different from normal [2].

Except its function as a cAMP-regulated chloride channel, CFTR has many other functions. CFTR can transport bicarbonate and may consequently regulate airway surface pH [8], which in turn may affect mucociliary clearance [9] and mucus viscosity [10]. However, there is no agreement on the pH of the ASL in CF patients in the literature [11-13].

Among the systems used to determine the composition of the ASL are cultures of airway epithelial cells. Although these would appear to be simple and well reproducible, the results obtained from these systems have been nearly as varied as those from more complicated

animal models [4, 5, 7, 14-16]. Most of these studies have been done on air-liquid interface cultures, in which the cells develop cilia and have a polarized phenotype close to airway epithelial cells *in situ*. However, Ehrhardt et al. [17] reported that the cell line 16HBE14o $^-$, a widely used bronchial epithelial cell line developed by Cozens et al. [18] only developed proper tight junctions when covered by fluid, i.e., under liquid-liquid culture conditions. This would make it improper to determine the composition of the ASL in air-liquid interface cultures.

In the present study, we used two new methods to determine the composition of the fluid covering the apical surface of airway epithelial cells in culture. We compared air-liquid with liquid-liquid culture conditions and normal airway epithelial cells with their CF counterparts. The large interest in CFTR-mediated HCO_3^- conductance and role of HCO_3^- in regulating airway ASL pH has led us to investigate the pH of the fluid covering the apical surface of airway epithelial cells.

Materials and Methods

Cell lines

16HBE14o $^-$ (16HBE) and CFBE41o $^-$ (CFBE) (homozygous for the ΔF508 mutation) cells were kind gifts of Dr. D.C. Gruenert, San Francisco [12]. The cells were grown in Eagle's Minimal Essential Medium (EMEM, SVA; Uppsala, Sweden) to confluence in tissue culture flasks. The confluent cells were trypsinized, split, and at least 1.6×10^5 cells were seeded on special inserts (Costar Transwell, Corning, NY, USA; 6.5 mm diameter, 0.4 μm pore size; 0.33 cm^2 growth area) on a tissue culture 24-well plate (Sarstedt, Newton, NC, USA; 1.2 cm diameter). The inserts had been coated with 100 μl of a solution containing 0.01% collagen type I from calf skin (Sigma, St. Louis, MO, USA). The collagen was allowed to bind overnight at 37°C. Excess fluid was removed from the coated surface, which was then dried for 30-60 min. The coated surface was sterilized by ultraviolet light for 2-3 h.

Some membranes with cells were chosen for analysis as soon as confluence was reached in liquid-liquid interface cultures (i.e., with growth medium both apically and basally). Other cultures were allowed to differentiate in air-liquid interface on the membranes for 3-4 weeks. For this, the medium was removed from the apical surface, and 700 μl of medium consisting of 48.5% Dulbecco's Modified Eagle's Medium (DMEM) with 1000 mg/l glucose (Gibco, Paisley, Scotland) 48.5% Ham's F12 medium with L-glutamine (Gibco), 2% Ultrosor G (Gibco), and 1% of a penicillin/streptomycin solution (Sigma, St. Louis, MO, USA) to give a final concentration of 60 $\mu\text{g/ml}$ penicillin and 50 $\mu\text{g/ml}$ streptomycin. No medium was added to the apical side of the culture but the culture was rinsed with 100 μl sterile isotonic phosphate-buffered saline (PBS) (140 mM NaCl, 5 mM Na_2HPO_4 , 4.2 mM KH_2PO_4) every other day.

The transepithelial resistance of both liquid-liquid and air-liquid cultures was measured with an EVOM epithelial voltmeter in an ENDOHM-6 chamber (World Precision Instruments, Sarasota, FL, USA). The measured transepithelial electrical resistance (TEER) in confluent cultures of 16HBE cells was about 1000 Ωcm^2 , and in confluent cultures of CFBE cells it was about 900 Ωcm^2 (not significantly different).

In the experiments where the cells were exposed to 10 mM NaCN to inhibit metabolic activity, cell viability was determined by double staining with propidium iodide and bisbenzamide H33342 (Sigma).

The pH measurements of the fluid covering the apical surface of airway epithelial cells in culture were done with a solid state PHR-46 Micro combination pH electrode (Lazar Research Laboratories, Inc., Los Angeles, CA, USA). For this series of experiments both cell lines under liquid-liquid conditions were used after 3-4 days of seeding. Medium from the apical surface was exchanged for a simple unbuffered solution, which did not contain HCO_3^- ions [10]. Liquid-liquid interface cultures were permitted to equilibrate with 10-50 μl of simple unbuffered solution [16], with or without added agonists or antagonists, or CFTR_{inh-172} [19], a specific blocker of CFTR (a kind gift of Prof. A. Verkman, Los Angeles, CA, USA) for 3h maximum. The pH measurements were done every hour until the tight junctions broke down in the unbuffered solution. Approximately after 3.5h the TEER in confluent cell cultures decreased to below 100 Ωcm^2 .

Frozen-hydrated samples

Liquid-liquid cultures of confluent 16HBE and CFBE cells were allowed to equilibrate with isotonic PBS for 24 h prior to the experiment. 7 μl of PBS was added to the apical surface of the cells, which gave a layer with a depth of about 200 μm . After 24 h the membrane was frozen in liquid propane cooled by liquid nitrogen to -190°C. The membrane was placed onto a specially designed holder and transferred to a Philips (Philips Electron Optics, Eindhoven, The Netherlands) 525 scanning electron microscope equipped with a Bio-Rad (Hemel Hempstead, UK) Polaron 7400E cold stage. The sample was coated with a thin carbon layer on the cold stage at a temperature of -190°C and kept at this temperature throughout analysis. X-ray microanalysis was performed at 20 kV for 100 sec with a beam size of 100 nm. For quantitative analysis, the data from the apical fluid were compared to results obtained from droplets of PBS, frozen and analyzed under the same conditions [20]. Air-liquid interface cultures of confluent cells were frozen (after four weeks of culture), transferred to the cold stage of the scanning electron microscope, and analyzed in the same way as described above for the liquid-liquid interface cultures.

Sephadex beads. Liquid-liquid interface cultures were allowed to equilibrate with 10 μl of isotonic PBS (with or without added agonists/antagonists) for 4-24 h as described above. Sephadex G-25 (diameter 20-40 μm) (Pharmacia, Uppsala, Sweden) beads were equilibrated for 10 min with the apical fluid in the following way. A small amount of beads was placed in the opening at the base of a Microlance 3 needle (0.8x40 mm; Beckton Dickinson, Dublin, Ireland); the needle was connected

Fig. 1. X-ray microanalysis of frozen hydrated apical fluid from 16HBE (n=14) and CFBE (n=12) cultures. Mean and standard error are given. Statistically significant differences are indicated by asterisks (*), ** p < 0.01.

to a syringe previously filled with air, and the beads were sprayed evenly over the airway surface by pressure on the syringe [21]. Sephadex is the trade name for a cross-linked dextran gel with ion-exchange capacity. Saturation of the beads with a salt solution is obtained after 5 minutes [22]. After absorption of the ASL, the beads were recovered by flushing with a hydrophobic volatile silicone oil (Dow Corning 200/1cS, DDH, Poole, UK) and collecting the beads in a watch-glass [23]. Under a preparation microscope, all adhering fluid was removed from the beads, and single beads were transferred onto specimen grids, which had been submerged into the oil. The specimen grids used were nylon electron microscopy grids (Agar Scientific, Stansted, UK). The grid with beads was slowly lifted out of the oil bath and mounted onto an aluminum holder covered with round carbon adhesive tape and left at room temperature for evaporation of the oil.

Sephadex beads were sprayed over the surface of the air-liquid interface cultures as described above and left there for 10 min. After that, they were recovered as described above.

Grids with Sephadex beads were carbon coated prior to analysis. X-ray microanalysis of the beads was carried out with the instrumentation described above, at 20 kV for 100 sec with a beam size of 100 nm. Typically 10-12 beads were analyzed from each sample. For quantitative analysis, the data were compared to the results obtained on beads soaked in salt solutions of different concentrations (50mM- 250mM) [21, 22, 24].

Statistics

Data are presented as mean $\pm$ standard error. Differences between more than two groups were determined by analysis of variance (ANOVA) and differences between two groups were determined using Student's t-test.

Fig. 2. Ionic composition (in mM) of the apical liquid from liquid-liquid interface cell cultures collected by Sephadex beads, as a function of the equilibration time of the apical fluid (4, 8, 24 h). Open bars: 16HBE, solid bars: CFBE, $n = 22-34$. Mean and standard error are given. Statistically significant differences between 16HBE and CFBE cells are indicated by asterisks (*), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Analysis of the apical fluid in frozen-hydrated specimens of liquid-liquid interface cultures showed that after equilibration for 24 h, the Na and Cl concentrations in the apical fluid had decreased, both in CF cells and in control cells, compared to the original PBS, in which the Na^+ concentration is 150 mM, and the Cl^- concentration 140 mM. The decrease was less in the CF cultures, and the Na and Cl concentrations were significantly higher in the apical fluid of CF cells compared to controls (Figure 1). Both in control cells and CF cells, the apical fluid was clearly hypotonic.

When the time of equilibration in liquid-liquid interface cultures was varied, and the ionic composition of the apical fluid was determined with the Sephadex bead method, the results showed that equilibration had taken place after 4 h, and that no further changes occurred after that point in time. At all three time points chosen, the apical fluid of CF cell cultures contained significantly more Na and Cl than control cultures but similar concentrations of K (Figure 2). Addition of 60 μM glibenclamide to control cell cultures resulted in an increase of Na, Cl and K concentrations in the apical fluid after 24 h (Figure 3). Glibenclamide did not have any effect on CF cell cultures (Figure 3). Exposure of control and CF cells to 10 mM NaCN, which inhibits cell metabolism and leads to a cell

Fig. 3. Effect of glibenclamide and NaCN on the ionic composition (in mM) of the apical liquid from liquid-liquid interface 16HBE and CFBE cell cultures collected by Sephadex beads. Mean and standard error are given, $n = 20$. Statistically significant differences from untreated cells are indicated by asterisks (*) for 16HBE, * $p < 0.05$ and ** $p < 0.01$ and significant differences between untreated control cells and CFBE cells are indicated by (#), ## $p < 0.01$. There is no significant effect of glibenclamide or NaCN in CFBE cells.

Fig. 4. Transmission electron micrograph of an air-liquid interface CFBE cell culture, showing ciliated cells. Bar = 2 μm .

death of about 40 %, resulted in significantly higher Na and Cl concentrations than in cultures not exposed to cyanide (Figure 3). The concentrations of Na and Cl as

Table 1. Effect of cAMP stimulation and a specific CFTR inhibitor on changes in pH (Δ pH) in the apical fluid in 16HBE cells and CFBE cells. The data are the average of 4-5 experiments.

Time	16HBE untreated	16HBE + forsk/IBMX	16HBE + CFTR _{inh172}	16HBE + forsk/IBMX + CFTR _{inh172}
1h	- 0.092	+ 0.133	- 0.70	- 0.032
2h	- 0.072	+ 0.143	- 0.57	- 0.032
3h	- 0.192	+ 0.100	- 0.55	+ 0.033

Time	CFBE untreated	CFBE + forsk/IBMX	CFBE + CFTR _{inh172}	CFBE + forsk/IBMX + CFTR _{inh172}
1h	- 0.033	+ 0.027	- 0.057	- 0.080
2h	- 0.037	- 0.026	+ 0.040	- 0.105
3h	- 0.107	- 0.037	+ 0.053	- 0.087

determined by the Sephadex bead method in liquid-liquid interface were somewhat higher than those determined by X-ray microanalysis of frozen-hydrated samples, but the fluid in control cultures was still clearly hypotonic.

When cells are grown at air-liquid interface, they develop cilia (Figure 4). Although no fluid is applied to the apical surface of the cell culture, an apical fluid layer produced by the cells appears. Analysis of frozen hydrated air-liquid interface cultures showed higher Na⁺ concentrations (134 vs 89 mM) as well as higher Cl⁻ concentrations (66 vs 40 mM) in the apical fluid of the CF culture compared to control, but also prominent peaks for the intra cellular elements P, S and K, indicating that the electron beam had completely penetrated the fluid layer and excited the underlying cells. Analysis of the fluid layer in air-liquid interface cultures sampled with the Sephadex bead method showed that it was hypotonic both in the control and the CF cultures, but that the concentrations of Na, Cl and K were significantly higher in the CF cultures (Figure 5). The ionic concentrations in the apical fluid of the air-liquid interface cultures collected with the Sephadex bead method were somewhat lower than in the liquid-liquid interface cultures.

pH measurements in the apical fluid of 16HBE cells, or CFBE cells, respectively, showed that under unstimulated conditions, the apical fluid of both the 16HBE cells and the CFBE cells became more acid from a starting value of 7.35 for both 16HBE and CFBE cells; when chloride efflux was stimulated with forskolin and IBMX, the apical fluid covering the 16HBE cells became more alkaline, whereas the fluid covering the CFBE cells still became more acid, indicating that CFTR transported bicarbonate in the wild-type cells, but not in the CF cells (Table 1). The alkalization of the apical fluid covering the 16HBE cells was blocked by the specific CFTR-blocker

Fig. 5. Ionic composition (in mM) of the apical liquid from air-liquid interface cell cultures collected by Sephadex beads. Open bars: 16HBE, solid bars: CFBE, n= 6. Mean and standard error are given. Statistically significant differences between 16HBE and CFBE cells are indicated by asterisks (*), * p < 0.05, ** p < 0.01, *** p < 0.001.

CFTR_{inh-172}, but no effect was noted when the inhibitor was added to CFBE cells (Table 1). Neither glibenclamide nor amiloride affected significantly the pH changes in the apical fluid in the absence of cAMP elevating agents (not shown).

Discussion

The data obtained in this study show (1) that the apical fluid covering cultured normal bronchial epithelial cells is hypotonic, (2) that there is no great difference

between air-liquid and liquid-liquid cultures in this respect, and (3) that the apical fluid covering CF cells has a significantly higher Na and Cl content than that of control cells.

The values obtained for Na and Cl concentrations on control cultures in the different experimental set-ups used in the present paper range from about 80 to 100 mM for Na⁺ and 60-80 mM for Cl⁻. (It should be noted that bicarbonate and organic anions are not measured by X-ray microanalysis). This is somewhat lower than the values obtained by Jayaraman et al. [16] who found values of 97 mM for Na⁺ and 117 mM for Cl⁻ in air-liquid interface primary cultures of bovine trachea. Our values are also lower than those obtained by Knowles et al. [14] in primary cultures from canine trachea (around 120 mM), as well as those found by Matsui et al. [5] and Tarran et al. [7] in primary cultures from human airway (125-140 mM), but higher than those obtained by Zabner et al. [4] in primary cultures from human airways (40-50 mM) and much higher than those obtained by McCray et al. [15] (18 mM Cl⁻) in primary cultures from mouse tracheal epithelial cells.

Apart from the difference in techniques to measure the composition of the apical fluid, an important difference between the present study and the studies referenced above is that those earlier studies used primary cultures of human and animal airway cells, whereas the present study used cell lines. The cell lines used in the present study are homogeneous and equivalent to absorptive surface epithelial cells [18]. The homogeneity of the system is an advantage, but also a limitation, since the data obtained may not be representative for the human airway *in situ*, where both glandular (secretory) and surface epithelial (absorptive) cells determine the composition of the ASL.

The studies in which the pH of the apical medium was determined give support to the notion that in the wild-type 16HBE cells, CFTR transports (some) bicarbonate ions, and that this transport can be inhibited by a specific blocker of CFTR; in CF cells, this transport is absent. The fact that amiloride had no significant effect on the pH of the apical fluid under unstimulated (basal) conditions, may be explained by a low activity of the Na⁺-H⁺ exchange mechanism if CFTR is not stimulated.

We could not find support for the notion that air-liquid cultures would be less suitable for these studies than liquid-liquid cultures [17]. In both cases, TEER values in excess of 300 Ωcm^2 were obtained, indicating that tight junctions had been formed. A hypotonic apical fluid can only be obtained if the paracellular pathway is closed,

since leakage of the isotonic medium from the basolateral compartment would increase the ionic concentrations in the apical fluid. In the apical fluid of the air-liquid cultures, the Na⁺ and Cl⁻ concentrations were less than in the liquid-liquid cultures; hence, there is no indication for increased leakage. In addition, we have in a previous study demonstrated tight junctions impermeable to lanthanum nitrate tracer in 16HBE14o⁻ cells under liquid-liquid culture conditions [25].

As possible reasons for the divergent results in the literature, Verkman et al. [2] cite the relatively small volume of ASL in air-liquid interface cultures, as well as the fact that some of the techniques used for measurement of the ionic concentrations in the ASL could disturb the epithelium. With liquid-liquid interface cultures the depth of the ASL is not a problem. The measurements on the frozen-hydrated apical fluid, while cumbersome, present an undisturbed system and show that the layer has sufficient depth so that the electron beam does not excite the underlying cells (this would show up as e.g., a peak for the main intracellular element phosphorus, but this peak is absent from the X-ray spectrum). The bead method could be suspected of disturbing the epithelium by contact of the beads with the cells, but if there is any effect of this, it appears to be minor. Also the difference between air-liquid and liquid-liquid cultures is minor, and all data point to the same result, namely that the ionic concentrations in the apical fluid of control cultures are lower than would be expected for an isotonic fluid.

The data of the present study show that the apical fluid of CF cultures has a higher content of Na and Cl than that of control cells. We have previously shown that the control 16HBE14o⁻ cell line has CFTR in its plasma membrane and has a cAMP-activated chloride transport mechanism, whereas the CF cell line CFBE41o⁻ does not transport chloride after stimulation with cAMP [26, 27]. Matsui et al. [5] did not find a significant difference in Cl⁻ concentrations between CF and control in primary cultures of human bronchial cultures, nor could McCray et al. [15] find such a difference in airway epithelial cell cultures from normal and CF mice. On the other hand, Zabner et al. [4] found that the concentrations of Na⁺ and Cl⁻ were twice as high in the apical fluid of CF cultures compared to control. Although our data agree with that study in so far that we also find increased Na⁺ and Cl⁻ concentrations, the difference between CF and control is less than that observed by Zabner et al. [4].

We also found that tracheal fluid in transgenic ΔF508 -CFTR mice had a significantly higher content of Na and Cl than tracheal fluid in control mice [28] and

that nasal fluid from CF-patients had a higher content of Na and Cl than nasal fluid of healthy controls, a difference that was in part due to the defective CFTR in CF-patients [29]. These findings are in line with studies by Reddy et al. [30] and Kunzelmann et al. [31] stating that activation of the epithelial Na⁺-channel (ENaC) requires activation of CFTR, hence in cultured CF cells, the activity of ENaC would be expected to be reduced.

Addition of glibenclamide, an inhibitor of CFTR, to the apical medium results in an increase of the Na⁺ and Cl⁻ concentrations over a 24 h period, strengthening the notion that in this absorptive cell model, inhibition of CFTR results in higher Na⁺ and Cl⁻ concentrations in the apical fluid. Also the data from the cells exposed to NaCN indicate that metabolically inactive or dead cells do not cause a decrease in the ionic concentrations of the apical fluid.

In summary, the paper presents two methods that can be used to determine the ionic composition of the ASL in cell cultures. The system is useful not only for

studies related to CF, but also for studies related to other airway diseases where changes in the ionic composition of the ASL are relevant [32]. In a model system consisting of absorptive surface epithelial cells, the apical fluid becomes hypotonic, and inhibition (by glibenclamide or NaCN) in control cells or absence of functional CFTR (in a CF cell line) results in slightly but significantly higher concentrations of Na⁺ and Cl⁻ in the apical fluid.

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