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Research Article

ALTERED PROPERTIES OF HUMAN AIRWAY EPITHELIAL CELL LINE CALU-3 WITH REDUCED CFTR FUNCTION

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Abstract

To understand the implications of the dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis (CF) airways, we generated Calu-3 cells with reduced CFTR function (Calu-3A) by stable transfection with anti-sense CFTR oligonucleotides and evaluated the antibacterial activity and protein secretions in apical surface fluid (ASF). Both Calu-3 and Calu-3A monolayers were cultured on permeable supports at an air-liquid interface and ASF were collected by washing the apical surfaces. Our results indicated that ASF of Calu-3A cells contained higher total protein concentration, reduced lysozyme secretion and reduced level of antibacterial activity compared to Calu-3 ASF. Calu-3A cells responded to the CFTR channel agonist, forskolin with decreased iodide-efflux and increased lysozyme secretion. These studies suggest that the decreased antibacterial activity of Calu-3A cells could be due to changes in ASF protein composition such as reduced lysozyme levels. The findings that decreased forskolin mediated iodide efflux, increased lysosyme secretion and decreased antibacterial activity of Calu-3A suggest altered physiological property of Calu-3A cells, implicating dysfunction of CFTR in CF airways.

Key words: Calu-3 cells, CFTR, lysozyme and antibacterial activity.

Introduction

The mechanisms of altered CFTR function leading to reduced antibacterial activity of CF airways remain incompletely understood. However, there is growing evidence to support a central role for CFTR in maintaining

respiratory tract homeostasis due to its role in fluid and electrolyte transport, protein secretion and mucin production in the airway [20,21]. Increasing evidence suggest that CFTR plays a role in airway protein and mucin secretion [7,11,12,16].

Human airway epithelial cell line Calu-3, which expresses high levels of functional CFTR has been extensively used in understanding the CFTR-mediated ion transport defect in CF [5,6,17]. It has also been shown that the antibacterial activity of ASF washings from Calu-3 cells grown at an air-liquid interface has a number of similarities to previously characterized antibacterial activity of ASF from primary cultures of airway cells [22]. Treatment of Calu-3 cells with ion-transport mediators resulted in altered protein secretion [7,15]. We have previously shown that forskolin and glybenclamide, which are known to modulate CFTR function, altered the Calu-3 secretion of total protein and lysozyme [16]. Another study suggested that pharmacological correction of the defective CFTR-mediated protein secretion is promising for the development of a rational drug therapy for CF patient [11]. Similarly, when rat submandibular cells were treated with anti-CFTR antibody, it inhibited β-adrenergic stimulation of mucin secretion [12].

In the present study we have made an attempt to assess the physiological responses of Calu-3 cells when CFTR function is defective. We evaluated the antibacterial and protein secretory properties in ASF of cells at an air-liquid interface culture. We tested the hypothesis that reduced level of Calu-3 CFTR function could result in altered Calu-3 ASF properties. To generate Calu-3 cells with reduced CFTR function (Calu-3A), we stably transfected Calu-3 with expression vectors that generate anti-sense CFTR mRNA. We then evaluated the effects of forskolin (CFTR channel agonist) and budesonide (inhaled corticosteroid used in CF patients) on Calu-3 and Calu-3A protein secretion. We further hypothesize that the alteration in antibacterial activity of Calu-3 and Calu-3A ASF could be due to altered protein secretion in the ASF.

Methodology

Generation of Calu-3A cells with reduced CFTR function

Calu-3A cells with reduced levels of CFTR protein was generated by expressing anti-sense mRNA [8,14,16,22]. Calu-3 cells were stably transfected with expression vector (pcDNA3.1) containing CFTR nucleotides 951 to 1850

in the anti-sense orientation. We used LipoTAXI mammalian transfection kit (Stratagene,La Jolla,CA) for transfection of Calu-3 cells and stable neomycin resistant clones with antisense mRNA were selected by RT-PCR. Both Calu-3 and Calu-3A cells were cultured at 37°C and 5% CO_2 in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) that was supplemented with 15% fetal calf serum (FCS), 500 U/ml penicillin and 50 µg/ml streptomycin. Cells were maintained in 75-cm^2 tissue culture flasks and split when 80-90% of confluency was reached. Culture medium within the insert (apical side) was removed on the second day after plating cells and medium was changed basolaterally on alternate days. Both Calu-3 and Calu-3A cells were cultured at 37°C and 5% CO_2 in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) that was supplemented with 15% fetal calf serum (FCS), 500 U/ml penicillin and 50 µg/ml streptomycin. Cells were maintained in 75-cm^2 tissue culture flasks and split when 80-90% of confluency was reached. Air-liquid interface culturing of Calu-3 cells was carried out by plating cells at 2 X 10^6 cells/cm^2 onto Costar transwell inserts (0.4 µm pore size, 12 mm diameter, clear polycarbonate membrane, Costar plates; Corning Inc., Corning, NY) that had been coated with human placental collagen. Culture medium within the insert (apical side) was removed on the second day after plating cells and medium was changed basolaterally on alternate days. After approximatley 11 days of culture at an air-liquid interface, confluent monolayers with a transepithelial resistance (TER) value of greater than 300 ohm·cm^2 were basolateraly treated with Forskolin (50 µM/ml) and air-liquid interface culture was continued. Cells were cultured for 24 hr, because under unstimulated conditions we expect a significant amount of fluid and protein accumulation by then [14,15]. Sham treated monolayers were used as controls.

Iodide efflux assay

The rate of I^- efflux measurements was done after stimulating cells with CFTR channel agonist forskolin (10 µM) [10]. Cells were incubated for 30 min with efflux buffer (in mM: 141 NaCl, 3KCL, $2KH_2PO_4$, 0.9 $MgCl_2$, 1.7$CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4) containing 5µCi/ml carrier-free ^{125}I (sodium salt). Extracellular ^{125}I was removed by washing four times with efflux buffer, and the rate of loss of intracellular ^{125}I was determined by replacing the bathing solution with 37^oC efflux buffer every 60s for 12 min.

Collection of ASF washings and antibacterial assay: Calu-3 and Calu-3A ASF were collected by washing the apical surface of each new monolayer three times with 60 µl of sterile water and combining the washes [16,22]. ASF washings were assayed at the time of collection or stored in aliquots (-20°C) until used in assays. For antibacterial assay, *Escherichia coli* M15 (E. *coli*) were washed three times with sterile, non-bacteriostatic water and resuspended to 10^4 or 10^6 cells/µl [22]. Serial dilutions were used to obtain samples with the desired number of colony-forming units (CFU) per microliter. In this fashion, known amounts of bacteria assayed by plating on luria broth (LB) agar plates were added to 30 µl of ASF washings and incubated overnight at 37°C. The bacteria-ASF mixture was then plated onto LB plates and CFU were counted after 24 hr.

Measurement of ASF lysozyme in Calu-3A ASF washings

Calu-3A cells were basolateraly treated with forskolin (50µM) for 24 hr and lysozyme levels in ASF washings was measured with a turbidimetric assay based on the enzymatic hydrolysis of *Micrococcus lysodeikticus* (*M. lysodeikticus*) cell walls and measuring a fall in optical density at 450 nm [16]. 20 µl of ASF washing was incubated in a suspension of *M. lysodeikticus* (0.3 mg/ml) for 18 hrs at 37° C. Lysozyme standards (0–500 ng/ml) and *M. lysodeikticus* suspensions were prepared in 50 mM sodium phosphate buffer pH 7.4 containing sodium azide (1mg/ml) and bovine serum albumin (1mg/ml). A standard curve was derived and the concentration of lysozyme in each sample was determined.

Measurements of ASF proteins

The total protein concentration was measured using a DC protein assay kit obtained from Bio-Rad Laboratories (Bio-Rad, Hercules, CA). The IL-8 kit (sensitivity 10 pg/ml) was obtained from R & D systems Inc. (Minneapolis,MN). The lysozyme kit (0.5 ng/ml) was obtained from ALPCO Diagnostics (Windham,NH). The values were measured and the concentration of IL-8 and lysozyme was normalized against the total protein of respective ASF washing.

Statistical Analysis

The protein values are expressed as mean ± standard error of the mean (S.E.M). One-way and repeated analysis of variance with post hoc analysis by the Student-newman-Keuls method was used with $P< 0.05$ considered significant.

Results

Calu-3 cell with the expression of neomycin resistant clone with anti-sense CFTR mRNA was confirmed by RT-PCR (Fig.1A) and cells with anti-sense CFTR expression were designated as Calu-3A. We examined the functional CFTR channel in Calu-3A cells by iodide efflux assay by treating cells with the CFTR channel agonist forskolin (10 μM). Our results indicated that forskolin significantly decreased I^- efflux in Calu-3A compared to Calu-3 cells, which indicated reduced level of CFTR activity in Calu-3A cells (Fig.1B).

Figure-1

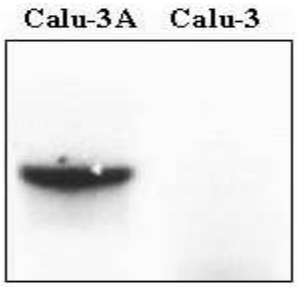

Figure-2

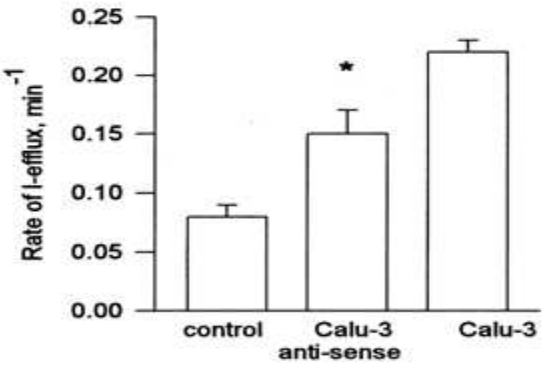

Figure 1: (A) CFTR anti-sense expression. Calu-3 cells were transfected with pcDNA3.1 expression vector containing CFTR nucleotides in the antisense orientation. Neomycin resistant clones were probed by RT-PCR for the expression of anti-sense mRNA and one clone with anti-sense mRNA as shown in the figure was designated as Calu-3A. (B) Forskolin-dependent Iodide (I^-) efflux. A significantly reduced level of CFTR activity was observed in Calu-3A cells as tested by I^- efflux assay (n=4; * $P < 0.05$ compared with Calu-3 cells). Cells were incubated with ^{125}I for 30 min and CFTR agonist forskolin (10μM) was added to all samples except the control. Time dependent rates of I^- efflux were calculated and maximal rates are plotted.

We next tested if Calu-3A cells respond to forskolin with altered lysozyme secretion, because we have previously observed a forskolin-dependent decrease in lysozyme secretion in Calu-3 cells [16]. In the present study Calu-3A cells exhibited a forskolin-dependent increase in lysozyme secretion as demonstrated by increased lysozyme level in ASF washings (Fig.2) as compared to sham treatment. In airways, loss of functional CFTR is known to cause reduced level of antibacterial activity. In these studies, we tested if Calu-3A cells with reduced CFTR function would also have reduced level of antibacterial activity.

Figure 2

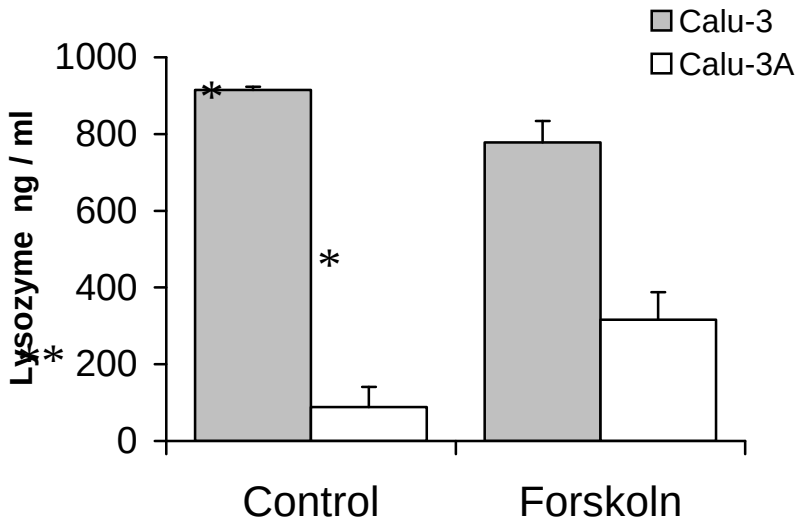

Figure 2: Secretion of lysozyme by Calu-3 and Calu-3A cells. Cells were treated with forskolin (50μM) for 24 hrs and apical surface fluid (ASF) washings were used for lysozyme assay by incubating with the suspension of M. *lysodeikticus*. Controls were sham treatment with sterile water. (n=10; ** P<0.001 compared with Calu-3 control. * P<0.01 compared with respective control).

To assess antibacterial activity in ASF washings of Calu-3 and Calu-3A, cells were grown at air-liquid interface culture and ASF washings were collected after 24 hr of culture. The ASF washings were collected and stored in aliquots for antibacterial activity assay and protein assay. The antibacterial activity was evaluated by treating ASF washings with E.coli. The ASF washings were incubated with an increasing amount of bacterial burden in log scale 1 to 10,000 of added colony forming units (CFU), after 24 hr Calu-3A washings were plated on LB agar plate and

incubated overnight at 37ºC and then CFU were counted. Fig.3 shows that CFU recovered in Calu-3A ASF washings was higher than CFU recovered from Calu-3 ASF washings, which indicated that Calu-3A ASF washings have reduced level of antibacterial activity.

Figure-3:

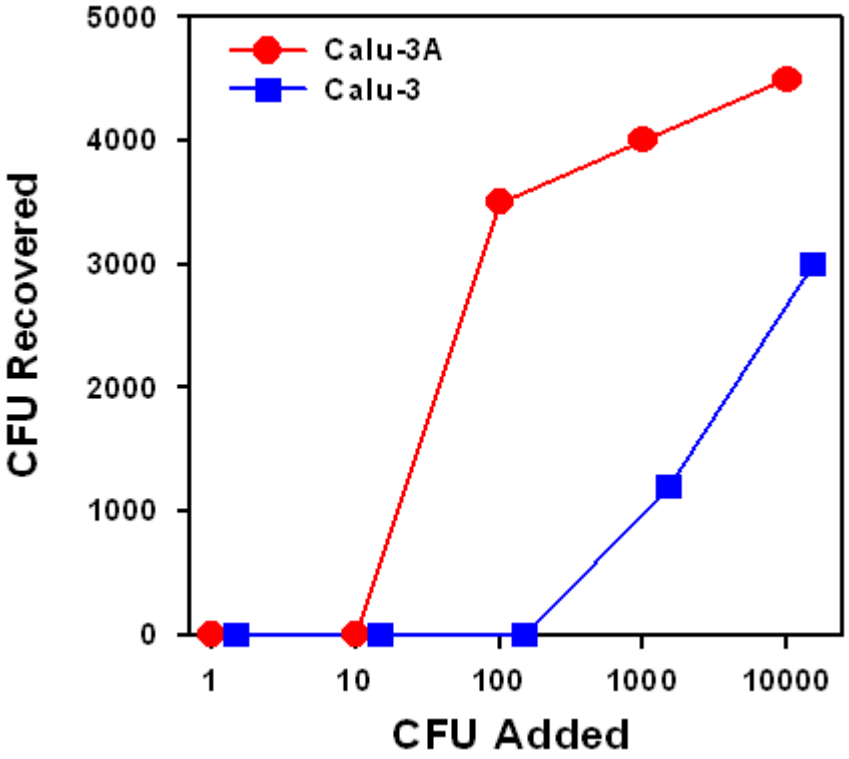

Figure 3: Reduced levels of antibacterial activity in Calu-3A ASF washings. The indicated number of *E coli* colony forming units (CFU) were added and incubated overnight in 30µl of ASF washings. Then ASF washings were plated onto LB agar and recovered CFU counted after 24 hr. Data are representative of 3 experiments for each inoculum of bacteria.

Discussion

There is growing evidence to support a central role for CFTR in maintaining respiratory tract homeostasis due to its role in fluid and electrolyte transport, protein secretion and mucin production in the airway [13, 14]. Calu-3 cells expresses high levels of functional CFTR, hence it has extensively been used in understanding the CFTR-mediated ion transport defect in CF [15-17]. At an air-liquid interface culture Calu-3 cells secrete ASF, which contained significant amount of *in vitro* antibacterial activity to bacterial strains commonly found in CF airways. It has been

proposed that human airway epithelial cell line Calu-3, grown at an air-liquid interface culture are a suitable *in vitro* model of the airway epithelium to study inhaled glucorticosteroid (budesonide) transport, absorption and local metabolism [20,21].

In the present study, Calu-3 cells with reduced level of CFTR function was demonstrated by the expression of anti-sense CFTR mRNA, the reduction of forskolin-mediated iodide-efflux, the increased level of forskolin-mediated lysozyme secretion and the reduction of the antibacterial activity. Forskolin, which activates CFTR through cAMP-dependent mechanisms, has been shown to increase bicarbonate secretion in Calu-3 cells [6,9]. Our results suggested that Calu-3A exhibited reduced level of forskolin mediated iodide-efflux, which is a characteristic of reduced CFTR function. Forskolin has also been shown to decrease lysozyme secretion in Calu-3 cells [16]. In the present study, we observed that forskolin increases Calu-3A lysozyme secretion, which indicated reduced level of CFTR function. In addition, the reduced level of antibacterial activity seen in Calu-3A ASF washings compared to Calu-3 ASF washings is analogous to the reduced antibacterial activity seen in CF airway disease [20,21].

Our results indicated that Calu-3 and Calu-3A cells exhibit different levels of total protein and lysozyme in ASF washings in sham treatment. Calu-3A exhibited an increased ASF total protein compared to Calu-3 ASF. However this increase was not due to increased lysozyme or IL-8 secretion, because Calu-3A exhibited significantly decreased lysozyme secretion in sham treatment, which might contribute to decreased level of antibacterial activity. In the human airway, the protein composition of ASF displays a remarkably high complexity and various antimicrobial mechanisms act in concert to protect the lung from infection [2,3,19,20,]. For example, the antibacterial activity of lysozyme and LL37/hCAP-18, a cathelicidin, have previously been reported to be synergistic *in vitro* [1].

We demonstrated above changes in protein secretion of Calu-3 and Calu-3A ASF, because these cells were capable of growing at an air-liquid interface culture, wherein cells were grown in direct contact with air, simulating *in vivo* condition of respiratory epithelium. Further the collection of ASF washings from apical surface enabled to evaluate the protein secretory properties of respiratory epithelium. In summary, we described the Calu-3 cell model with

defective or reduced CFTR function. The decreased antibacterial activity of Calu-3A cells could be due to changes in protein content such as reduced lysozyme levels in ASF. The above Calu-3 cell model will be useful not only in evaluating airway protein secretions and also in understanding the defective CFTR function in CF airways.

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