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Research Article

Human airway epithelial cells investigated by atomic force microscopy: A hint to cystic fibrosis epithelial pathology

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ABSTRACT

The pathophysiology of cystic fibrosis (CF) airway disease stems from mutations in the *CF Transmembrane Conductance Regulator* (*CFTR*) gene, leading to a chronic respiratory disease. Actin cytoskeleton is disorganized in CF airway epithelial cells, likely contributing to the CF-associated basic defects, i.e. defective chloride secretion and sodium/fluid hypersorption. In this work, we aimed to find whether this alteration could be pointed out by means of Atomic Force Microscopy (AFM) investigation, as roughness and Young's elastic module. Moreover, we also sought to determine whether disorganization of actin cytoskeleton is linked to hypersoption of apical fluid. Not only CFBE41o- (CFBE) cells, immortalized airway epithelial cells homozygous for the F508del *CFTR* allele, showed a different morphology in comparison with 16HBE14o- (16HBE) epithelial cells, wild-type for *CFTR*, but also they displayed a lack of stress fibers, suggestive of a disorganized actin cytoskeleton. AFM measurements showed that CFBE cells presented a higher membrane roughness and decreased rigidity as compared with 16HBE cells. CFBE overexpressing wtCFTR became more elongated than the parental CFBE cell line and presented actin stress fibers. CFBE cells absorbed more fluid from the apical compartment. Study of fluid absorption with the F-actin-depolymerizing agent Latrunculin B demonstrated that actin cytoskeletal disorganization increased fluid absorption, an effect observed at higher magnitude in 16HBE than in CFBE cells. For the first time, we demonstrate that actin cytoskeleton disorganization is reflected by AFM parameters in CF airway epithelial cells. Our data also strongly suggest that the lack of stress fibers is involved in at least one of the early step in CF pathophysiology at the levels of the airways, i.e. fluid hypersorption.

1. Introduction

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein is a cAMP-activated ion channel directly involved in the transport of chloride and participating in the transport of other ions, namely sodium and bicarbonate, in absorbing/secreting epithelia of many organs [1,2]. Indeed, the CFTR protein is expressed by the sweat gland, trachea, lung, pancreas and several tissues of the reproductive system [1–4]. Cystic fibrosis (CF) is the most lethal autosomal recessive genetic disorder in the Caucasian population and is due to mutations in the *CFTR* gene, located on chromosome 7. To date, more than 2000 mutations have been identified in the CFTR gene (http://www.genet.sickkids.on.ca/app), although for many of them it is not known at the moment the pathogenic effect [5]. These mutations have been classified in six different classes [6], with the most common mutation being a 3 base deletion, that induces a deletion of a phenylalanine at position 508 (F508del), found in approximately 66% of all CF chromosomes [7]. Although many organs are affected by the diseases, the chief cause of morbidity and mortality of CF individuals is due to the lung disease, whose hallmarks are recurrent opportunistic bacterial infections and a deregulated neutrophil-dominated chronic inflammation.

In epithelial cells, apical localization of CFTR protein and its chloride channel activity strictly depend on interactions of its PDZ (for postsynaptic density 95/disclarge/zona occludens)-interacting domain in the C terminus with other proteins primarily localized to the apical surfaces [8,9]. Thus CFTR localization to the apical plasma membrane and its function are regulated by these

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macromolecular complexes, involving F-actin, the scaffolding protein Na^+/H^+ exchange regulatory factor 1 (NHERF1) and ezrin [10]. We have previously reported that while NHERF1 and the active form of ezrin are mainly localized in the apical region in non-CF airway epithelial cell monolayers, they are diffusively expressed in the cytosol of CF airway epithelial cells. Moreover in CF cells, the actin cytoskeleton is disorganized, compared to non-CF cells [11]. More recently, we have also shown that in comparison to non-CF cells, CF cells have a reduced localization of zonula occludens (ZO-1) and other proteins at the level of tight junctions TJs [12], and a correspondingly higher permeability to dextrans, indicating a disorganization of (TJs), coupled to the lack of actin stress fibers. Others have also reported that lack of a functional CFTR on the CFBE plasmamembrane is coupled with deranged transcellular and paracellular permeabilities [13–16].

Recently, the possibility of using some nanomechanical parameters and morphological characteristics, achieved by means of Atomic Force Microscopy (AFM) measurements, as an indication of cellular disease has largely increased because the obtained results seem to be very interesting. Indeed, AFM has been widely applied in single cell studies because such technique yields valuable results by scanning or indenting a cell surface with a very weak force (of the order of nN or pN). In fact, it has been reported that cellular elasticity, described by the values of the Young modulus, is related to the structure of actin filaments below the cellular membrane [17] and it is modified during the evolution of a pathological condition [18]. In particular, it is well known that tumor cells are significantly softer than normal cells [19–21] and that the acquisition of invasive capabilities corresponds to a lowering of Young modulus values, as reported for fibroblasts [20], for tongue carcinoma cells [21], and for oral squamous cell carcinoma [22]. In addition, it was also demonstrated that the final step of metastatic colonization in melanoma process corresponds to a stiffness value increasing and becoming larger than in normal cells [23]. The cellular Young modulus values are influenced also by pathologies different from tumor ones: a decrease of stiffness in myoblasts with increasing of oxidative stress induced by hydrogen peroxide exposure [24], an increase of the stiffness of vascular smooth muscle cell in hypertension disease [25], and an increase of adipocytes stiffness in adipose-related disease [26] were also reported. Another nanomechanic cellular parameter (evaluable through AFM measurements) whose values are related to disease stage is cell roughness. Smooth cell surface profiles for non-cancerous cells and rough cellular morphology of cancerous cells have been measured [27], as well as alterations of membrane roughness of red blood cells from individuals with anemia disease have been found [28]. Nonetheless, the roughness parameters are certainly less widespread than elasticity ones for characterizing the evolution of cell disease, because they need to be evaluated after a proper filtering procedure of measured data in order to achieve accurate results [29].

The study of the basic defects in the airway epithelium associated with CF has took much advantage from the creation of suitable cells lines from non-CF and CF lung samples. The most used cell lines are those produced by Dr. D. Gruenert, namely 16HBE14o- (16HBE) cells obtained from a non-CF subject [30], and CFBE41o- (CFBE) cells, homozygous for the F508del allele [31]. Their status in CFTR biogenesis and expression is adherent to cystic fibrosis genetics and pathogenesis: while 16HBE cells express CFTR protein in both mature and immature forms in the cytoplasm and in the mature form only in the membrane [32], CFBE cells, being homozygous for the F508del mutation, do not express CFTR on the plasmamembrane and forskolin has no effect on Cl^- ion transport in these cells [33]. These cell lines have been useful in characterizing many aspects of CF-associated alterations related to epithelial biology, including vectorial ion transport [31–33], CFTR regulation by phosphodiesterases [34], and inflammatory response [35]. Very few reports have been generated on the evaluation of stiffness and Young's modulus in airway and respiratory epithelial cells [18,36], and none on the differences between bronchial/bronchiolar epithelial cells obtained from non-CF and CF individuals. In this paper, we sought to analyze these mechanical properties in well-characterized 16HBE and CFBE cell lines used in the CF field by AFM and combining these data with standard cell biology techniques. We found that CF cells display a higher stiffness and lower Young's modulus which may be correlated with a disorganized actin cytoskeleton.

2. Materials and methods

2.1. Cell cultures and optical microscopy

16HBE14o- cells and CFBE41o- cells were obtained from Dr. D. Gruenert, University of California, San Francisco, CA. 16HBE14o- cells are SV40 large T-antigen immortalized bronchial epithelial cells that have been described as being derived from a 1-yr-old male heart-lung transplant patient [30]. CFBE41o- cells are also SV40 immortalized bronchial epithelial cells (1st bifurcation). They derived from a female CF patient homozygous for the F508del mutation [37,38].

Cells were grown in Eagle's minimal essential medium (MEM; Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin (PAA Pasching, Austria) at 37 °C under 5% CO_2. Cells were routinely grown on plastic flasks coated with an extracellular matrix containing fibronectin/vitrogen/bovine serum albumin. The extracellular matrix coating was prepared as follows: 10 mg/ml fibronectin adhesion-promoting peptide (BD Biosciences, Milan, Italy), 100 mg/ml albumin from bovine serum (Sigma-Aldrich), and 30 mg/ml bovine collagen type I (BD Biosciences) were dissolved in MEM. The mixture was sterilised by 0.2-μm filter.

A number of 3×10^5 16HBE or CFBE cells were seeded onto glass coverslips which were coated with fibronectin/vitrogen/bovine serum albumin for 5 h. Twenty-four hours later, cells were fixed in 3% paraformaldehyde (PFA), 2% sucrose for 10 min. Optical images were collected by means of an inverted microscope (Leica DM IRB) operating in the phase contrast mode.

2.2. Transfection and flow cytometry

A number of 5×10^4 cells were plated on 6 wells plates and transfected with 1 μg plasmid pS65T carrying the wild-type CFTR cDNA under the control of the CMV early enhancer/promoter region (a kind gift from Bruce Stanton, Dartmouth Medical School, Hanover, USA) [39] by Lipofectamine® 2000 (Thermo Fisher) according to the manufacturer's instructions. To analyze the percentage of cells transfected, 5 days later cells were detached, fixed in 3% PFA and 2% sucrose in PBS and permeabilised with ice-cold Triton HEPES buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) for 5 min at room temperature; then cells were incubated with CFTR antibody MAB 25031 mouse IgG2a (R&D Systems, Minneapolis, MN) at 1:20 dilution for 1 h at 4 °C. After washing in PBS, the cells were incubated with the TRITC-conjugated secondary antibody (anti-mouse used at 1:100; Sigma-Aldrich) for 1 h at 4 °C. After two washes in PBS, cells were resuspended in 50 μl PBS, counterstained with DAPI and analyzed by Amnis Flowsight IS100 (Merck Millipore). Brightfield aspect ratio versus brightfield area plots were generated to identify single cells events, then 10,000 single-cell events for sample were acquired. The percentage of positive cells (channel 3,488 nm excitation laser) was analyzed using