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Modelling the bronchial barrier in pulmonary drug delivery: A human bronchial epithelial cell line supplemented with human tracheal mucus

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ABSTRACT

The airway epithelium together with the mucus layer coating it forms a protective system that efficiently filters and removes potentially harmful particles contained in inhaled air. The same mechanism, however, serves to entrap particulate drug carriers, precluding their interaction with their target. The mucus barrier is often neglected in *in vitro* testing setups employed for the assessment of pulmonary drug delivery strategies. Therefore, our aim was to more accurately model the bronchial barrier, by developing an *in vitro* system comprising a tight epithelial cell layer which may be optionally supplemented with a layer of human tracheal mucus. To form the epithelium *in vitro*, we used the cystic fibrosis cell line CFBE41o-, which can be grown as monolayers on Transwell® supports, expressing tight junctions as well as relevant transport proteins. In contrast to the cell line Calu-3, however, CFBE41o- does not produce mucus. Therefore, native human mucus, obtained from tracheal tubes of patients undergoing elective surgery, was used as a supplement. The compatibility of CFBE41o- cells with the human mucus was addressed with the MTT assay, and confirmed by fluorescein diacetate/propidium iodide live/dead staining. Moreover, the CFBE41o- cells retained their epithelial barrier properties after being supplemented with mucus, as evidenced by the high trans-epithelial electrical resistance values (~1000 Ω cm^2) together with a continued low level of paracellular transport of sodium fluorescein. Fluorescently-labeled chitosan-coated PLGA nanoparticles (NP, ~168 nm) were used as a model drug delivery system to evaluate the suitability of this *in vitro* model for studying mucus permeation and cell uptake. Comparing CFBE41o- cell monolayers with and without mucus, resp., showed that the NP uptake was dramatically reduced in the presence of mucus. This model may therefore be used as a tool to study potential mucus interactions of aerosolized drugs, and more specifically NP-based drug delivery systems designed to exert their effect in the bronchial region.

1. Introduction

The conducting airways of the lungs are coated with a viscoelastic secretion, the pulmonary mucus, which moisturizes the inhaled air and acts as a filter for inhaled particles. In the healthy state, mucus is composed of water (95% w/w), glycoproteins (mucins, 2–5%), salts, non-mucin proteins, lipids, DNA, enzymes, cells and bacteria [1–3]. The mucins are continuously secreted into the airway lumen by specialized secretory cells, and polymerize to form a mesh-like structure that is constantly being propelled out of the lungs by the ciliary beating of the airway epithelial cells – this creates a dynamic barrier termed as mucociliary clearance [4–6]. In disease states such as asthma, chronic obstructive pulmonary disease (COPD), and in particular cystic fibrosis (CF) considerable changes in the mucus can occur, leading to mucus oversecretion and mucus thickening [7–10]; this in turn can compromise the mucus clearance mechanism, providing optimal conditions for bacterial growth and chronic infection [11].

CF is a lethal genetic disease caused by a mutation of the CF transmembrane conductance regulator (CFTR). This results in numerous irregularities including an abnormal hydration of the airways, which leads to an impairment of the mucociliary machinery, recurrent infections, and eventually premature death [11]. The clinical management of CF focuses primarily on improving the mucociliary clearance and combating chronic infections rather

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than targeting the primary cause, namely correcting the genetic disease. The potential of nanomedicine to improve the efficiency of gene-therapy in such diseases by using nanoparticle (NP)-based drug delivery systems is considerable, as evidenced by efficient transfection of cell-based *in vitro* models including the CF cell line CFBE41o- [12–14]. This cell line was generated by transformation of CF airway cells with the SV40 virus and is homozygous for the most common CF mutation, the F508-CFTR mutation. Of particular interest to pharmaceutical research is the ability of this cell line to express tight-junction proteins such as claudin-1, ZO-1, and occludin [15], which confer on CFBE41o- monolayers significant epithelial barrier properties evidenced by high transepithelial electrical resistance (TEER) values [15–18]. This cell line also expresses a number of proteins relevant for pulmonary drug transport, including P-glycoprotein (P-gp), lung resistance-related P protein (LRP), and caveolin-1 [15]. Unfortunately, unlike other pulmonary cell lines such as Calu-3, which are able to secrete mucus [19–21], the CFBE41o- cell line lacks the capacity to synthesize and secret mucus onto the cell monolayer – a key feature that must be taken into account in the context of airway research. In particular, with regard to the use of NP-based drug delivery systems to treat bronchial diseases, our work and that of others has previously shown that particles with a diameter above 200 nm are almost exclusively trapped within the pulmonary mucus [3,22,23]. Moreover, with a net negative charge under physiological conditions [24,25], mucus represents a significant barrier to positively charged nanocarriers [21,26], which are often used in the context of nucleic acid delivery for transfection purposes [27–29].

Therefore, in the present study we explored the possibility to develop an *in vitro* model of the airways composed of a CFBE41o- cell layer coated with human tracheal mucus. Our aim was to take a step forward in accurately mimicking the scenario within the CF lung, by utilizing the positive features of the CFBE41o- cell line in the context of pharmaceutical research and further introducing mucus as a key non-cellular barrier of the airways. For this purpose we cultured CFBE41o- cells in Transwell® supports and added a layer of human tracheal mucus on top of the cell monolayer, creating an air-mucus interface. The biocompatibility of CFBE41o- cells with the human tracheal mucus was investigated by measurement of epithelial barrier properties upon incubation with the exogenous mucus. Ultimately, as a proof-of-concept validation of the implemented *in vitro* model, we produced chitosan-poly(D,L-lactide-co-glycolide; PLGA) nanoparticles and determined the effect of the mucus layer on the cellular uptake of such particles.

2. Methods

2.1. Human mucus sample collection

Undiluted human tracheal mucus samples were collected by the endotracheal tube method [3,30,31], after obtaining informed consent from patients and in compliance with a protocol approved by the Ethics Commission of The Chamber of Medicine Doctors of the Saarland (file number 19/15). The tracheal tube of patients undergoing elective surgery with general anesthesia, non-related to pulmonary conditions, was collected after surgery. The distal portion of the tracheal tube (5–10 cm), including the balloon, was cut and placed in a 50 ml centrifuge tube. The mucus of each tracheal tube was collected by centrifuging the samples at 190*g* for 30 s. Samples with visible blood contamination were excluded from the analysis. Mucus samples were stored at −20 °C until further use. In total 16 mucus samples from independent patients were used in this study. The mean age of the patients was 56.8 ± 4.8 years, the male: female ratio was 12:4, and 6 out of 16 patients were smokers.

2.2. Freeze-dried mucus disk preparation

Mucus samples frozen and stored at −20 °C were thawed gradually and allowed to reach room temperature. Thereafter, single mucus drops with an approximate weight of 30–40 mg (34.17 ± 1.82 mg, n = 45 mucus drops) were placed over a Teflon® surface and spread over delineated circular surfaces of 1.12 cm^2. The samples were then placed into an autoclavable sealing bag, stored at −80 °C for 4 h, and ultimately, freeze-dried overnight (Alpha 2-4 LSC, Christ, Germany). After completion of the preset freeze-drying program, the bag containing the mucus disks (1.7 ± 0.1 mg estimated solid content, for an estimated water content of 95%) was immediately sealed and stored in a dry atmosphere at room temperature until further use. Five different batches with 14–20 mucus disks per batch were used in this study.

2.3. Mucus characterization

2.3.1. Mucus bulk rheology

Experiments were conducted on an Anton-Paar MCR 102 rheometer (Graz, Austria) equipped with cone-plate geometry (diameter: 25 mm, cone angle: 2°) at room temperature. Strain amplitude (γ) sweeps were performed at a frequency of 1 Hz in the range of 0.1–10%. Frequency (ω) dependency of the storage modulus G′ and the loss modulus G″ was measured in the range between 0.1 and 40 rad/s at a strain amplitude of 1%.

In the first set of experiments native undiluted tracheal mucus samples were gradually thawed and allowed to reach room temperature. Thereafter, an approximate volume of 150 µl of mucus was placed in the rheometer and the aforementioned protocol was conducted. In the second set of experiments previously freeze-dried and rehydrated mucus samples were analyzed. Mucus samples contained in 1.5 ml Eppendorf tubes were allowed to equilibrate to room temperature and weighed using a precision balance (CPA 224S, Sartorius, Göttingen, Germany). Afterwards, the samples were stored at −80 °C for 4 h followed by overnight freeze-drying (Alpha 2-4 LSC, Christ, Osterode am Harz, Germany). The freeze-dried (solid) content of the samples was weighed again to determine the water content of mucus. Mucus samples were then re-hydrated with exactly the same volume of sublimed water (Milli-Q water, Advantage A10, Merck Millipore, Billerica, MA), and were allowed to mix in a 360° multi-rotator (PTR-35, Grant instruments, UK) for at least two hours at room temperature. Thereafter, re-hydrated mucus samples were placed in the rheometer to perform the measurements as described above.

2.3.2. Scanning electron microscopy

The structure of pulmonary mucus was imaged by means of scanning electron microscopy (SEM). Human tracheal mucus samples were gradually thawed and spread over the surface of a SEM-imaging carbon disk. The mucus was freeze-dried *in situ* following the freeze-drying protocol as in Section 2.2. Freeze-dried mucus samples were gold-sputtered (QUORUM Q150R ES, Gala Instrumente, Germany) and then transferred to the SEM (EVO HD15, Zeiss, Germany) for imaging.

In order to image the CFBE41o- cell monolayer and the combined model comprising the cell monolayer and the overlying mucus, the cells were seeded onto Transwell® permeable supports and were cultured until a confluent monolayer was reached (see Section 2.4). The day before the SEM fixation the apical culture medium was removed and a mucus disk together with 100 µl of fresh medium were added to the apical compartment, creating an air mucus interface. The cells with the mucus disks in place were incubated for 24 h at 37 °C, 5% CO_2, in a horizontal shaker. After incubation the basolateral medium was aspirated and fixation was performed by adding 1 ml of glutaraldehyde 3% (Sigma)