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The characterization of the human cell line Calu-3 under different culture conditions and its use as an optimized *in vitro* model to investigate bronchial epithelial function

Mateja Erdani Kreft [a], Urška Dragin Jerman [a], Eva Lasič [a], Neli Hevir-Kene [b], Tea Lanišnik Rižner [b], Luka Peternel [c], Katja Kristan [c,*]

[a] Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia
[b] Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia
[c] Lek Pharmaceuticals, d.d., Sandoz Development Center Slovenia, Verovškova 57, 1526 Ljubljana, Slovenia

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ABSTRACT

In this study we have investigated the effects of different cell culture conditions on the Calu-3 epithelial cell model. Calu-3 cells were cultured in media A-MEM at the air–liquid (A–L) or liquid–liquid (L–L) interface for one or three wks (weeks). Different cryomethods were tested and the cell line was characterized using histochemistry, immunofluorescence, transmission and scanning electron microscopy, transepithelial resistance (TEER) measurements, permeability studies, and gene profiling of 84 drug transporters. Cell culture was successful in A-MEM with only 2.5% FBS. Cell proliferation and viability depended on the cryopreservation method. All Calu-3 models expressed CK7, occludin, and E-cadherin. The A–L interface resulted in a more biomimetic native bronchial epithelium displaying pseudostratified columnar epithelium with more microvilli and secretory vesicles than at the L–L interface, where the epithelium was cuboidal, but exhibited higher TEER values and lower dextran permeabilities. Longer time in culture significantly decreased dextran permeability and increased the expression of specific drug transporters. Drug transporter expression was also notably influenced by the culture interface, where the A–L interface yielded a higher expression of drug transporter genes than the L–L interface. Since cell culture interface and time in culture affect Calu-3 cell differentiation, barrier integrity, permeability properties, and drug transporter expression, culture conditions need to be considered and standardized when using the Calu-3 cell line as an *in vitro* model for aerosol drug delivery and screening of bronchial drug candidates.

1. Introduction

Airway epithelium is gaining attention as a site for drug delivery as well as to study drug toxicity. The administration of aerosol therapeutics is a non-invasive method with the advantages of a large absorptive surface area, leaky epithelium, and extensive vascularization, as well as avoiding first-pass metabolism, intestinal efflux transporters, and degradation in the digestive system (Audus et al., 1990; Ehrhardt et al., 2008; Wengst and Reichl, 2010). Furthermore, the pulmonary route is favorable for pulmonary diseases, allowing local drug delivery (Foster et al., 2000). However, because the airway epithelium is a protective interface, its permeation properties must be assessed in order to evaluate its suitability for drug delivery (Fulcher et al., 2005). To date, not enough attention has been given to cellular mechanisms involved in drug permeation in airway epithelia and its drug transporters remain to be thoroughly characterized.

The properties of airway epithelium relevant to drug delivery are cell differentiation, formation of a polarized tight cell layer, production of mucus, development of cilia, and expression of transporters and metabolic systems (Florea et al., 2003). To study airway epithelium, *in vitro* models have been utilized using excised tissue, primary cell cultures, or immortalized cell lines (Wengst and Reichl, 2010). However, excised human tissue is hard to obtain

Abbreviations: ABC, ATP-binding cassette; AQP, aquaporin; A–L, air–liquid interface; BSA, bovine serum albumin; FBS, fetal bovine serum; L–L, liquid–liquid interface; SEM, scanning electron microscopy; SLC, solute carrier; TEER, transepithelial electrical resistance; TEM, transmission electron microscopy; TJ, tight junction; P_{app}, apparent permeability coefficient.

* Corresponding author. Tel.: +386 1 580 3461; fax: +386 1 568 35 17.
E-mail addresses: mateja.erdani@mf.uni-lj.si (M.E. Kreft), urska.dragin@mf.uni-lj.si (U.D. Jerman), eva.lasic@mf.uni-lj.si (E. Lasič), neli.hevir-kene@mf.uni-lj.si (N. Hevir-Kene), tea.lanisnik-rizner@mf.uni-lj.si (T.L. Rižner), luka.peternel@sandoz.com (L. Peternel), katja.kristan@sandoz.com (K. Kristan).

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and using animal tissues introduces problematic species differences (Merkle et al., 1998). On the other hand, *in vitro* cell cultures increase the possibility of using human tissue and reduce time-consuming, expensive, and controversial animal studies in addition to offering quick permeability modeling (Audus et al., 1990). Primary cell cultures have disadvantages such as limited access to tissues, complex isolations, short lifespans, considerable heterogeneity within and between cultures, and uncertain reproducibility (Merkle et al., 1998; Wengst and Reichl, 2010) and the financial expenditures of human airway epithelial cells often hinder their use in experimentation (Fulcher et al., 2005). On the other hand, immortalized cell lines exhibit genetic homogeneity, provide reproducibility, and are easily maintained in culture (De Fraissinette et al., 1995; Wengst and Reichl, 2010; Werner and Kissel, 1996).

The Calu-3 cell line demonstrates many characteristics of bronchial epithelium and has become a popular model used to study anion secretion (Devor et al., 1999; Lee et al., 1998; Shan et al., 2011; Shen et al., 1994), transcytosis of dimeric immunoglobulin dIgA (Loman et al., 1997), mechanisms of allergenic proteinases (Winton et al., 1998), cellular responses to oxygen- and ventilator-induced injury (Zhu et al., 2010) and virus infections (Harcourt et al., 2011), functional barrier properties of bronchial epithelium (Cooney et al., 2004; Fiegel et al., 2003; Grainger et al., 2006; Singh et al., 1997; Wan et al., 2000), and drug transporters (Endter et al., 2009; Hutter, 2012; Mukherjee et al., 2012). The Calu-3 cell line has been found to be the most appropriate cell line for studying bronchial epithelium, due to its closest resemblance to this epithelium *in vivo*. Only Calu-3 cells express the cystic fibrosis transmembrane conductance regulator, (cAMP)-dependent Cl^- secretion, form tight polarized monolayers with TJs and appreciable TEER values, and display microvilli (Shen et al., 1994), as well as expressing the lung surfactant-specific protein proSP-C (Fiegel et al., 2003) and containing mucin granules (Kreda et al., 2007). Additionally, Calu-3 cells grow rapidly and consistently (Foster et al., 2000) and can be reliably used over a wide passage range (Hutter, 2012; Shen et al., 1994).

However, culture conditions significantly affect cell differentiation and this can lead to much variability, and hence the characterization of epithelial cells under different culture conditions is crucial. Cell culturing techniques for the Calu-3 cell line vary, yet their standardization would be valuable if this cell line is to be used as a standard model for bronchial epithelium. For this reason, we have further characterized and optimized the Calu-3 cell line model using different culturing conditions, histochemical assessment, immunolabeling of protein expression, ultrastructural analysis using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), TEER measurements, permeation studies, and screening for the expression of various drug transporters.

2. Materials and methods

2.1. Cell Culture

Calu-3 cells were obtained from the American Type Culture Collection (LGC Standards, Cat No. ATCC-HTB-55, Lot No. 58052345) and used from passages 19 to 42. Calu-3 cells were maintained in A-MEM (Advanced Minimum Essential Medium, Gibco) supplemented with 4 mM GlutaMAX™ and 2.5% FBS. The cell cultures were maintained at 37 °C in a >95% humidified atmosphere of 5% CO_2 in air with media changes on alternate days. The cells were harvested when 80–100% confluent with TripLE™ Select (Gibco), collected, and centrifuged at 200*g* for 5 min. The cell pellet was resuspended in culture medium, the cells were counted with a haemocytometer and their viability assessed with the Trypan blue method. Cells were seeded at densities from 6×10^4 cells/cm^2 to 1×10^6 cells/cm^2 onto polystyrene Tissue Culture Flasks (25 cm^2, TPP) and cell culture growth and viability was assessed.

For the evaluation of Calu-3 cell models on different substrates and interfaces, the cells were seeded onto three different polyethylene terephthalate porous membranes with pore sizes of 0.4 μm or 1 μm (BD Falcon Cat. No. 353090, 353180; Corning, HRS Transwell-24-Systems Cat. No. 3379; and Milipore, Millicell®-24 Cat. No. PSRP 010 R1) at seeding densities from 1×10^5 to 1×10^6 cells/cm^2. To evaluate the effect of time in culture as well as submerged culture conditions or air interface on Calu-3 cells, cells were seeded onto porous membranes with the established optimal density of 4×10^5 cells/cm^2 and culture medium was added to both the apical and basal compartments for the liquid (L–L) interface, whereas for the air–liquid (A–L) interface the culture medium was removed from the apical compartment after 24 h. Calu-3 epithelial models on porous membranes were maintained in culture for one or three weeks (wks).

2.1.1. Assessment of cryopreservation techniques

When the Calu-3 cells reached confluence in a 75 cm^2 Tissue Culture Flask (TPP), they were harvested as described above and resuspended into a cryovial (TPP) in either FBS:DMSO (9:1) or FBS:A-MEM:DMSO (2:7:1) at densities of 2×10^6 cells/mL or 5×10^6 cells/mL. They were transferred to 4 °C for 1 h, then to −20 °C for 3 h, and finally stored at −80 °C or in liquid nitrogen at −196 °C. After three wks the cells were quickly thawed in a water bath at 37 °C, centrifuged at 200*g* for 5 min, and resuspended in culture medium. The cells were counted and their viability assessed with the Trypan blue method. They were then seeded onto Tissue Culture Flasks (TPP) at a density of 8×10^4 cells/cm^2 and their proliferation was assessed.

2.2. Transepithelial electrical resistance (TEER) measurements

TEER was measured using an epithelial voltohmmeter (EVOM, WPI) and electrodes (STX2, WPI) when the Calu-3 cells at the L–L and A–L interfaces reached confluence on the porous membranes with 0.9 cm^2 effective growth area (BD Falcon) and 0.4 μm pore size. Culture media (0.5 mL) was added to the apical compartment of the A–L interface to conduct the measurements. TEER was measured for three wks; $n = 6$ for each interface. The measured TEER values were corrected by subtracting the mean resistance of blank porous membranes (150 Ω cm^2).

2.3. Histology and histochemistry

The Calu-3 cells were seeded with a density of 4×10^5 cells/cm^2 and cultured at either interface on porous membranes (BD Falcon, 0.4 μm pore size) for one or three wks. Afterwards they were fixed in 10% formalin overnight at 4 °C. After dehydration and paraffin embedment, 5 μm sections were prepared with a Microtome (Leica RM 2135), stained in haematoxylin and eosin, Periodic Acid Schiff stain (Sigma) for neutral proteoglycans, or Alcian blue (Sigma) for acid proteoglycans, and viewed with a light microscope Nikon Eclipse TE300.

2.4. Immunofluorescence

The Calu-3 cells were seeded with a density of 4×10^5 cells/cm^2 and cultured on porous membranes (BD Falcon, 0.4 μm pore size) for one or three wks. Then they were fixed in ice cold absolute ethanol for 25 min at room temperature and then washed with phosphate-buffered saline (PBS) for 15 min. The cells were first blocked with 1% bovine serum albumin (BSA) in PBS, then incubated overnight at 4 °C with the primary antibodies rabbit polyclonal anti-