Calu-3 Model Under AIC and LCC Conditions and Application for Protein Permeability Studies

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Abstract

Broad area of respiratory epithelium with mild surface conditions is an attractive possibility when trans-mucosal delivery of protein drugs is considered. A mucus and cellular barrier of respiratory epithelium can be modelled *in vitro* by Calu-3 cell line. We have monitored morphology and barrier properties of Calu-3 culture on permeable supports while developing into liquid covered or air interfaced and mucus lined cellular barrier. Besides morphological differences, cultures differed in electrical resistance and permeability to proteins as well. The accelerated permeability to proteins in these models, due to permeability modulator MP C16, was examined. The effect on electrical resistance of cellular layer was rapid in both cultures suggesting easy access of MP C16 to cells even though its overall impact on cell permeability was strongly reduced in mucus covered culture. Differences in properties of the two models enable better understanding of protein transmucosal permeability, suggesting route of transport and MP C16 modulator action.

Keywords: Calu-3 cells, Proteins, Pulmonary drug delivery, Epithelial permeability, Macromolecular drug delivery, Absorption enhancer

1. Introduction

Transmucosal delivery of macromolecular drugs is frequently considered as a desired alternative to less convenient subcutaneous injections. Delivery of low molecular mass drugs through intestinal mucosa is the most common and well accepted route of drug administration. However, it exhibits hardly to overcome obstacles to rapidly expanding group of protein therapeutics. Harsh intestinal conditions can readily disable protein therapeutics. Tight intestinal mucosa is a potent permeability barrier to large hydrophilic molecules, while its modulation may result in inflammatory response.

Respiratory epithelium is not as readily accessible as intestinal; on the other hand its luminal surface represents much milder environment lacking huge amounts of proteolityc enzymes, extreme pH conditions and foreign substances or microbes. Additional advantages of intranasal and intrapulmonary drug administration over the oral one are rapid absorption kinetics and avoidance of the first-pass metabolism.[1–3] The applicability of respiratory route for systemic delivery of proteins was confirmed in the case of insulin[4] so it encourages the search for novel approaches of delivery through respiratory mucosa.

In vitro cell culture models have been extensively used to study transmucosal transport as well as drug metabolism and toxicity effects. Calu-3 is mucus secreting cell line originating from a lung adenocarcinoma.[5] These cells are suggested to be of serous origin and in culture express mixed phenotype consisted of ciliated and secretory cells. Calu-3 cell model was shown to form confluent monolayer of well differentiated cells with suitable barrier function and several functional characteristics of native respiratory epithelium.[6–9] Due to such properties it represents the most frequently used cell culture respiratory epithelium model. These cells have been widely used as a model of cystic fibrosis,[10,11] to study electrolite transport,[12,13] toxicity of drugs and their metabolism[7,14] as well as for assaying of permeability to low and large molecular substances.[7,9,15,16]

Among larger molecules peptides, dextrans, insulin and immunoglobulin permeation was examined.[9,17–19,20] Overall there is a lack of data about Calu-3 epithelium model permeability to other pharmaceutically important proteins. To overcome low permeability of epithelia to large molecules various permeability accelerators were suggested. Out of them alkyl maltopyranosides are among most potent. Their activity is believed to be detergent nature related. Common structure of the group is maltose linked to an alkyl chain of varying length. The representative of 16 carbon atom alkyl chain, N hexadecyl-β-D maltopyranoside (MP C16) ($C_{28}H_{54}O_{11}$) is one of largest in the group studied so far. Its effect on TEER values and permeability were demonstrated before to be achieved at low doses.[21]

An ability of Calu-3 cells to build up mucus layer in air interfaced culture (AIC) as a contrary to liquid covered culture (LCC) enables creating representative model of respiratory mucosa covered with mucus or the one with cellular layer only. Here we present development of both Calu-3 cell line based respiratory epithelium models and compare their permeability to few model proteins of therapeutic interest in order to elucidate the contribution of cellular and mucus layer to the epithelial barrier. The results suggest little mucus related barrier function for our model proteins. The protein transport enhancement as well as an effect on TEER in the presence of epithelium permeability modulator MP C16 was less pronounced in AIC model suggesting interference of the modulator with mucus.

2. Experimental

2. 1. Materials

Cell line Calu-3 was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Cell medium components, minimum essential medium eagle (MEM), Dulbecco's modified eagle's medium (DMEM), heat inactivated foetal bovine serum, L-glutamine, non-essential amino acids were from Sigma-Aldrich (St Louis, MO). Antibiotic/antimycotic solution was from Invitrogen (Carlsbad, CA). Permeability studies were done on Transwell® (Costar, Cambridge, MA) polyester permeable supports (diameter, 12 mm; pore size 0.4 μm). Permeability markers of low molecular mass (propranolol hydrochloride, ranitidine hydrochloride and fluorescein sodium) were obtained from Sigma-Aldrich (St Louis, MO). Insulin was from Novo Nordisk Pharmaceuticals (Bagsvaerd, Denmark), granulocyte colony-stimulating factor (G-CSF) and tumour necrosis factor-alpha (TNF-α) were prepared in-house, and erythropoietin (EPO) was a gift from Lek d.d. (Ljubljana, Slovenia). Protein purity was confirmed for all proteins used by SDS-PAGE; and Novex® Colloidal Blue staining (Invitrogen, Carlsbad, CA). Antibodies for ELISA were from R&D Systems (Minneapolis, MN). A permeability modulator MP C16 was obtained from Affymetrix (Santa Clara, CA). Alcian blue for mucus staining was from Sigma.

2. 2. Cell Culture

Cell line Calu-3 was cultured in T flasks at 37 °C in growth medium composed of MEM supplemented with 10% (v/v) heat inactivated foetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) non-essential amino acids in an atmosphere containing 5% CO_2. Cells were subcultured once a week before reaching 90% confluence. For the transport experiments, cells between the passage numbers 23–33 were used. Permeable supports were seeded at a density of 200,000 cells per Transwell® insert. They were grown in medium DMEM containing 10% (v/v) foetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) antibiotic/antimycotic, being replaced every 2–3 days. Liquid covered culturing (LCC) was changed in cultures dedicated for air interfaced culturing (AIC) after day 2 in a way that apical side was rinsed during regular medium replacement and left medium free. Transport experiments were performed after 14 days culturing on inserts.

2. 3. Transepithelial Electric Resistance (TEER)

TEER values were monitored regularly before routine medium replacement with a Millicell-ERS volt-ohm meter (Millipore, Billerica, MA). Measurements were performed in triplicate at room temperature. TEER values were not corrected for the resistance of the cell-free filters (which is approximately 150 Ωcm^2). TEER was measured before and after each permeability experiment. When TEER was reduced during the transport experiment, it was monitored for a further 24 hours to evaluate regeneration of cell monolayers in a fresh medium.

2. 4. Scanning Electron Microscopy

Calu-3 cells cultured on inserts for selected time were rinsed with phosphate and cacodylate buffer followed by 45 minute fixation in cacodylate buffer containing 1% glutaraldehyde and 1% formaldehyde, adjusted to pH 7.2 from apical and basal surfaces. After fixation the epithelium was bathed for 5 subsequent 3-minute periods in cacodylate buffer. It was followed by 30 minutes incubation in cacodylate buffer containing 1% osmium tetraoxide in dark. After this the washing in cacodylate series was repeated and dehydration of epithelium was carried out in progressively increasing concentrations of ethanol to be finally processed in critical point dryer. Supporting membranes holding dried epithelium were then excised from inserts and mounted on specimen stubs with adhesive carbon tape and platinum coated using Bal-Tec sputter coater (SCD 050). Cells were analyzed at 3 kV with FE-SEM JSM-7500F (JEOL, Japan).

2. 5. Mucus Staining

Calu-3 cells grown under AIC and LCC conditions were rinsed with PBS buffer and then fixed in 5% paraformaldehyde solution for 15 minutes at 37 °C. After another washing in PBS cells were incubated in alcian blue solution (pH 2.5) for 60 minutes and subsequently destained in the MiliQ water. Caco-2 cells were stained by the same procedure and used as a negative control. Mucus staining on Calu-3 and Caco-2 cell models was compared under the light microscope.

2. 6. Permeability Experiments

Permeability experiments were performed on inserts holding 14-day-old cultures of Calu-3. Culture medium was replaced with DMEM on basal (1.5 ml) and apical (0.5 ml) side of cells after gentle rinsing with DMEM to remove debris and serum. Substances to be tested were diluted in DMEM and applied to the apical side of cell culture to make final 0.5 ml. Samples (110 μl) from the basal side of culture were collected 30, 60, 120 and 180 minutes after treatment. The sampling volume was immediately replaced by fresh DMEM. The final sampling was done also at the apical compartment to check stability of tested compounds. TEER value control to check integrity of the cell monolayer was done as described above.

2. 7. Analytical Procedures

Concentrations of tested substances in samples from permeability experiments were determined by fluorimetry, ELISA or HPLC. Fluorescein amount was determined by microplate reader (FLUOstar Galaxy, BMG Labtechnologies GmbH, Ortenberg, Germany). Excitation and emission wavelengths were 485 and 530 nm respectively. Concentrations of EPO, TNF-α and G-CSF were determined by in-house sandwich ELISA based on commercial antibodies and reagents. Concentrations of insulin were determined with ELISA kit IS130D (Calbiotech, San Diego, CA) according to the manufacturer's protocol. Propranolol and ranitidine contents were analysed by RP-HPLC (Alliance 2695, Waters, Milford, MA) with an YMC-Pack ODS-AQ using a C18, 150 × 4.6 mm, 3 μm ID column. The mobile phase for propranolol was 0.05% phosphoric acid in 25% acetonitrile. UV detector 2487 was used at 292 nm for detection. For ranitidine, the mobile phase consisted of 0.05 M ammonium acetate and acetonitrile (75:25). The absorbance was measured at 313 nm.

Trend lines for increasing sample concentrations in a basal compartment as a function of time were used to calculate apparent permeability coefficients (P_{app}) according to the equation: $P_{app}=dQ/(dt\,A\,Co)$, where dQ/dt is the permeability rate, *A* is the diffusion area of the monolayer, and C_0 is the initial concentration of the tested sub-

stance in the apical compartment. All permeability experiments were performed in three repeats.

2. 8. Toxicity Experiments

The toxicity assessment of MP C16 for Calu-3 cells was based on living cells dependent enzymatic conversion of methyl thiazole tetrazolium (MTT) substrate. Calu-3 cells were seeded on microtiter plates and grown to confluence. Cells were then treated with selected concentrations of MP C16 (0.0025%, 0.0075% and 0.0125%) for 3 hours as in the permeability experiment, followed by medium replacement and addition of MTT substrate (Sigma-Aldrich, St Louis, MO) to final 1mg/ml immediately or after 24 hours regeneration. After 3 hours of incubation all liquid was discarded and formazan product dissolved in DMSO. Absorbance at 570 nm was measured by microplate reader (FLUOstar Galaxy, BMG Labtechnologies GmbH, Ortenberg, Germany). The viability of cells after three hours exposure or one day after treatment was expressed as the percentage of untreated control.

2. 9. Statistical Analysis

Data are presented as means of repeated experiments with corresponding standard errors (SEM). Where responses were illustrated by representative experiments, averages and standard deviations are presented (SD). Significances of changes were determined by student's t-test with p values being below 0,05.

3. Results and Discussion

3. 1. Development and Evaluation of Calu-3 Epithelium Model in LCC and AIC Conditions

The Calu-3 cell line based respiratory epithelium model has been used before to study permeability to low molecular mass substances.[7,16,22] Its ability to form mucus layer in AIC conditions promises more complex model better reflecting conditions *in vivo*. Therefore we decided to take a closer look into both, LCC and AIC Calu-3 models to point out their characteristics during culturing time related development and to compare their permeability to large molecules as well as their response to a permeability enhancer.

To compare Calu-3 cell cultures grown under LCC and AIC conditions TEER values were monitored regularly over two week culturing period on permeable supports. Cell proliferation was comparable in both cultures as assumed from growth area coverage time. TEER values were increasing steadily until day 6 or 7 when maximal values were met (Fig 1). Later TEER remained constant or it was slightly decreasing. The values for AIC model were in a range between 300 and 600 Ωcm². In the LCC

model TEER values were always higher (700–1000 Ωcm^2) and generally more subjected to fluctuations. The difference in final TEER between AIC and LCC models is substantial, indicating important differences in a cell layer, the principal barrier component. TEER values were
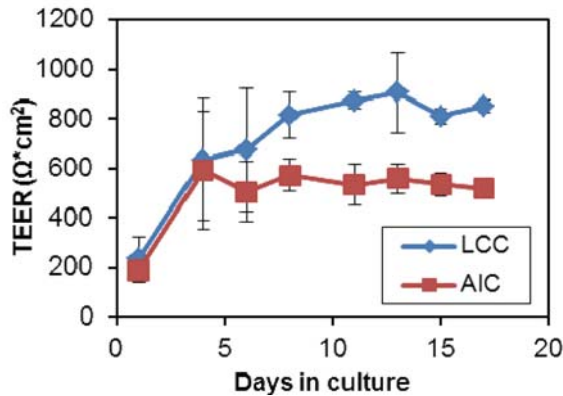
generally up to 2 fold higher in LCC model which is in accordance with previous reports.[18,23,24]

The Calu-3 cells in culture exhibit certain features of *in vivo* respiratory epithelium. Shortly after reaching confluence, surface mucus accumulation in the AIC model could be observed by bright field microscopy. Sparsely at the beginning it expanded all over the cells in next days. Presence of mucus in AIC was confirmed through high affinity staining with alcian blue. On the other hand staining in LCC was evidently less intensive but still slightly more prominent than in no mucus producing cell line (Fig 2A) which may indicate traces of mucus. It is not clear whether culturing conditions regulate mucus expression or mucus is dissolved in cell culture medium in LCC and removed by regular medium exchange. Most researchers report about the presence of mucus in the AIC model[9,18,25] although there are also some reports about mucus formation in LCC conditions.[17,26] The mass overlaying cells in AIC could be additionally detected in scanning electron microscope preparations when repetitive rinsing during preparation was omitted (Fig 2B).

Scanning electron microscopy was also used for close insight into morphological differentiation from the time
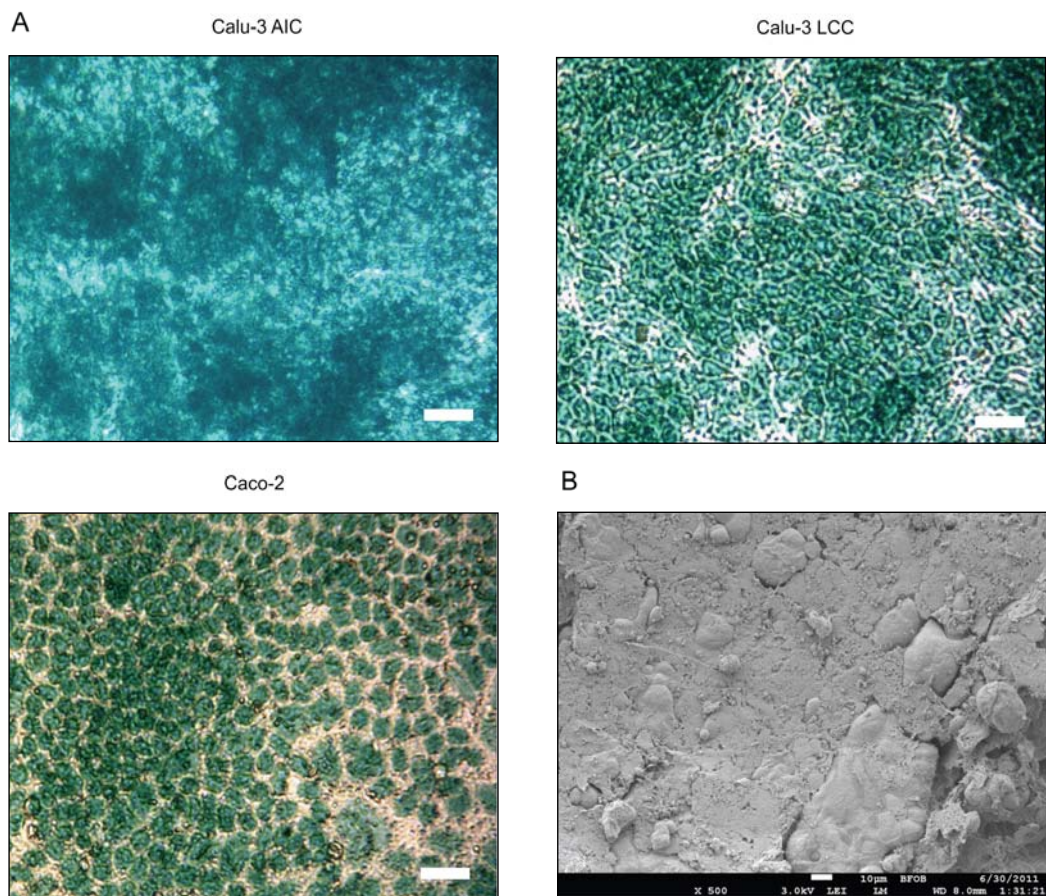

Figure 1. Calu-3 epithelium model formation. The transepithelial electrical resistance across Calu-3 cell cultures as a function of time in LCC and AIC conditions. Values from three representative experiments are shown as an average ±SEM.

Figure 2. Mucus formation in air interfaced culture of Calu-3 cells. (a) Light microscopy of Calu-3 (AIC and LCC) and Caco-2 cell cultures after alcian blue staining. Representative images from one out of three independent experiments are presented. White bars represent 50 μm. (b) Scanning electron micrograph of Calu-3 culture in air interfaced condition. Extensive rinsing was omitted to preserve mucus layer. White bar represents 10 μm.
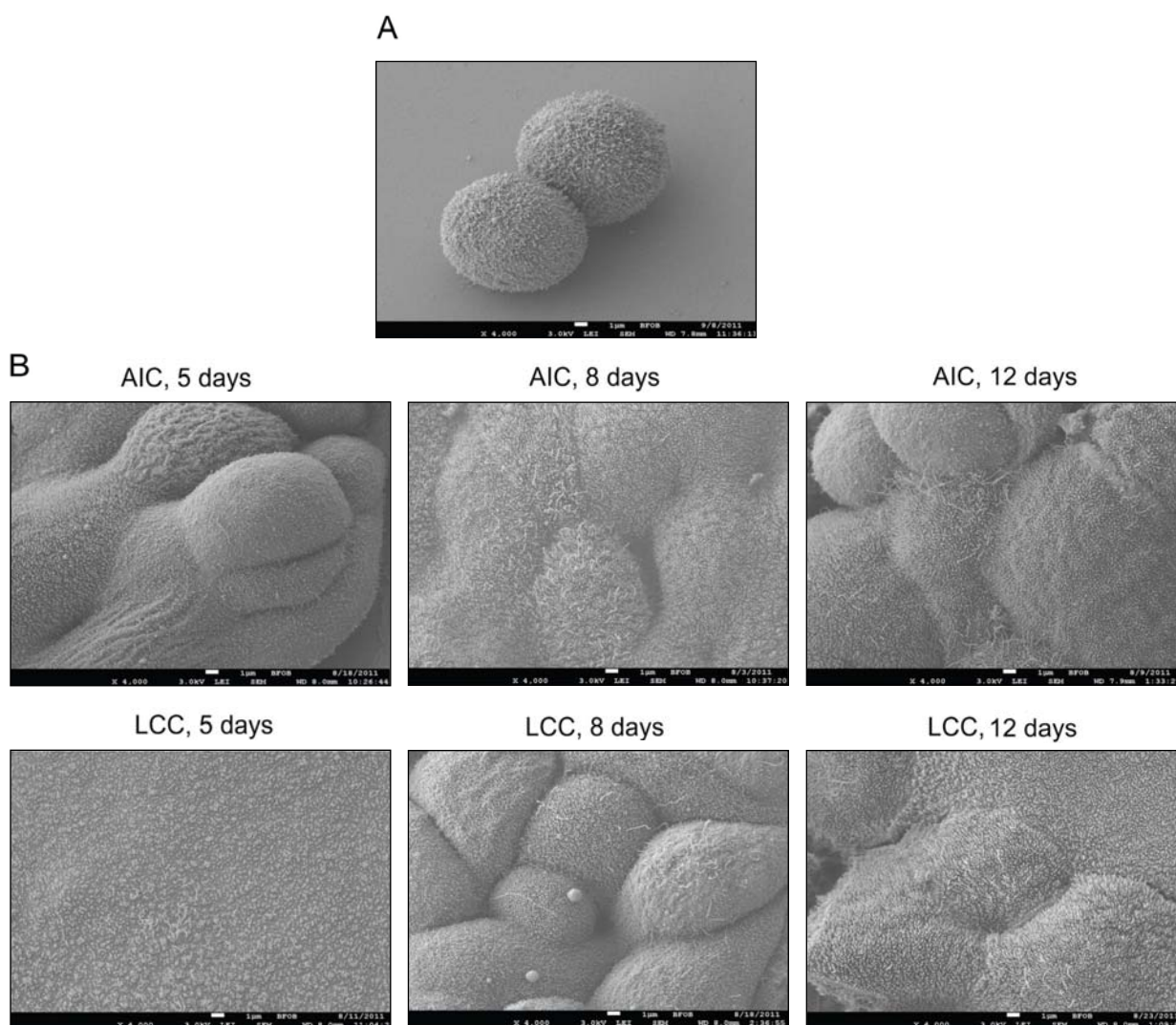
Figure 3. Scanning electron micrographs of Calu-3 cultures. Detached cells before seeding on permeable supports (a) and cells after 5, 8 and 12 days on permeable supports under AIC and LCC conditions (b). Magnification 4000×. White bars at the bottom of micrographs represent 1 µm.

of seeding till final development associated with LCC and AIC culturing of Calu-3 cells. It was shown that trypsinized cells before seeding on permeable supports already exhibited surface microvillus structures. This is a plausible reason for rapid formation of brush border like apical surface after subculturing. The rapid reset of apical microvillus structure is also concomitant with the fast reach of maximal TEER value. Microvillus structures were very short before seeding cells on permeable supports and after attachment of cells they became progressively denser and longer. These features seem to be more pronounced in AIC conditions. In AIC some protrusions resembled cilia, according to their outstanding length. The differences among cells, and sometimes within individual cell apex were however substantial. Representative images are compiled in figure 3.

Another difference in morphology between the two models was observed. Bright field microscopy revealed uniform monolayer in LCC, while more heterogeneous appearances were observed in AIC. Further z-stack analysis under the confocal microscope confirmed formation of simple monolayer in the case of the LCC model while in AIC model cell nuclei were positioned at different levels, resembling pseudostratified morphology of the native respiratory epithelium (data not shown). On the other hand, some cell agglomerations and multilayering could be detected in the AIC model by scanning electron microscopy. Microscopy methods thus revealed cell agglomerations and mucus accumulation in AIC model resulting in a thicker barrier while in LCC model cells were arranged in a uniform single layer. Microscopy and TEER data therefore suggest better cell to cell assembly under LCC conditions limiting diffusion of ions between apical and basolateral side to a higher extent than under AIC conditions.

The AIC and LCC models were further evaluated for permeability to low molecular mass substances. At first

the barrier function was tested with fluorescein. The overall permeability was similar as published previously with P_{app} values about 1×10^{-7} cm/s,[9,16,25] but it was regularly about two fold higher in AIC than in LCC. Transition rate of ranitidine, another paracelluar permeator, was also two fold higher in AIC over LCC model with P_{app} values of 6×10^{-7} and 3×10^{-7} cm/s respectively. On a contrary permeation to lipophilic propranolol, a good transcellular permeator was found to be tenfold higher in LCC, the culture lacking hydrophilic mucus lining and with cells organised strictly in a simple monolayer (Table 1).

Table 1. Permeability of Calu-3 based LCC and AIC models to low molecular weight markers. Permeability to fluorescein, ranitidine and propranolol is presented with P_{app} values. Values are means ± SEM of three independent experiments. Permeability values differed significantly for ranitidine and propranolol.

Compound	P_{app} (cm/s) – LCC	P_{app} (cm/s) – AIC
Fluorescein	$8.5 \pm 3.9 \times 10^{-8}$	$1.5 \pm 0.8 \times 10^{-7}$
Ranitidine	$3.0 \pm 0.9 \times 10^{-7}$	$6.4 \pm 1.6 \times 10^{-7}$
Propranolol	$8.7 \pm 1.4 \times 10^{-6}$	$9.0 \pm 0.8 \times 10^{-7}$

3. 2. Protein Permeation

Permeation of the Calu-3 epithelium models to proteins was tested using four model proteins of pharmaceutical interest. The permeation rates of EPO, G-CSF, TNF-α and insulin were low when compared to low molecular mass markers (Table 2). They varied four- to five folds among each other but P_{app} values for all proteins were generally a few fold higher in AIC than in LCC conditions in all individual experiments; however, due to high interassay variability significance was confirmed only in EPO where higher number of repetitions were performed (Figure 4 and Table 2).

Permeability to proteins therefore largely followed that of small paracellularly transported molecules and ions. Lower TEER values and higher permeability of small hydrophilic molecules suggest less restricted paracellular route in AIC. On a contrary, transcellular permeability in AIC was reduced which could be attributed to thickened cell layer and mucus lining. According to this proteins seem to be transported predominantly by the
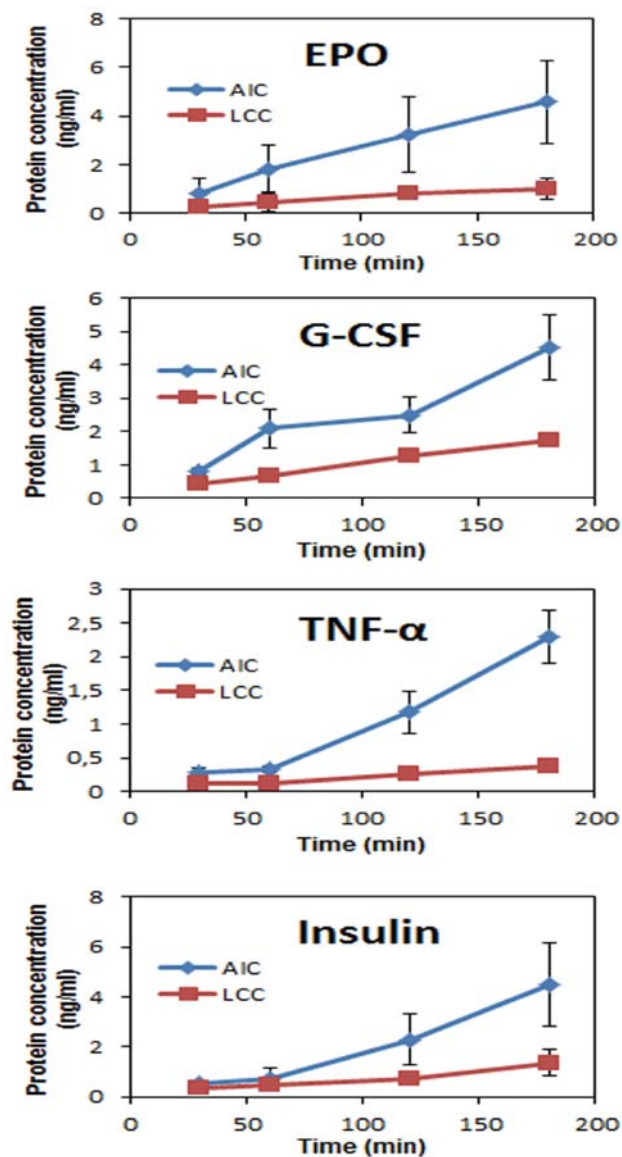

Figure 4. Apical to basal transport of model proteins across the Calu-3 based epithelium models. Concentrations of EPO, TNF-α, G-CSF and insulin in a basal compartment are plotted against incubation time over LCC and AIC Calu-3 models. Data are an average of three repeats in one representative experiment ± SD. Permeability values (Papp) obtained from all independent experiments are presented in Table 2.

paracellular pathway which is consistent with the conclusions by Pezron et al.[17] about the insulin transport across the Calu-3 model. P_{app} values for insulin on our model were comparable to previously reported for Calu-3 cells.[16,19,27] Insulin is a small protein when compared to most others therefore we compared it to three other proteins for which we couldn't find any data about Calu-3 permeability in the present literature. Certain differences in permeability among selected proteins could be determined in both models; however, differences could not be related to molecular masses only. More detailed research

Table 2. Permeability of Calu-3 based LCC and AIC models to four proteins. Permeability to EPO, TNF-α, G-CSF and insulin is presented with P_{app} values. Values are means ± SEM (n = 2–11). Permeability values in LCC and AIC culture differ significantly for EPO.

Protein	P_{app} (cm/s) – LCC	P_{app} (cm/s) – AIC
EPO	$1.4 \pm 1.9 \times 10^{-9}$	$5.0 \pm 3.0 \times 10^{-9}$
TNF	$4.2 \pm 0.6 \times 10^{-10}$	$2.4 \pm 1.1 \times 10^{-9}$
G-CSF	$5.4 \pm 0.1 \times 10^{-10}$	$1.2 \pm 0.9 \times 10^{-8}$
Insulin	$4.8 \pm 4.0 \times 10^{-10}$	$6.7 \pm 4.8 \times 10^{-9}$

could perhaps reveal the contribution of individual protein features. Differences in cell layer of the two models disable exact evaluation of mucus contribution to overall barrier function in AIC, however low TEER values and higher permeability for hydrophilic molecules speak for little contribution in this respect. Recently Vlasalieu et al.[20] showed much higher insulin permeability being comparable to values we and others obtained for small molecules like fluorescein.[9,25] Their data suggest dextrans permeability to be highly mucus dependent but less depending on TEER value. Different macromolecular permeability may be related to type of molecule. Semi-linear dextrans may interact with mucus differently than globular proteins. Besides, in our AIC culture N-acetyl cysteine had no obvious effect on mucus coverage and permeability results, suggesting differences in mucus layer (data not shown). It has been shown before that similar models may perform differently in different laboratories; however, we and many others found clear dependence of paracellular permeation on TEER value of epithelium model.

Alkyl maltopyranosides are a group of nonionic surfactants which were shown before to enhance epithelium permeability to different compounds.[28–30] We studied permeability response of AIC and LCC models to a long chain representative of alkyl maltopyranosides, MP C16 ($C_{28}H_{54}O_{11}$). It consists of hydrophilic maltose glycosidically linked to a hydrophobic 16-carbon alkyl chain, and is one of the less characterized in literature. It was chosen for its low critical micelle concentration (CMC) value (0.00003%) at which modulator effect on epithelium barrier is to be expected. Its effect on TEER values and permeability of a protein on both models was measured. The epithelium modulating effect of MP C16 was found to be rapid and reversible. In treated models rapid decrease in TEER to final values in both LCC and AIC condition was observed. In 15 minutes TEER already dropped almost to the lowest value where it remained until removal of MP C16. The TEER decrease was dose dependent; however, it was far more pronounced in LCC and regeneration of TEER after medium exchange was successful only at lower concentrations (Fig 5). This modulator effect was less prominent in AIC where the same concentrations of MP C16 had milder impact on TEER values and regeneration after treatment period was more successful as well. The decrease in TEER values and their regeneration in AIC resembled those in LCC treated with lower concentration of MP C16.

We conclude that mucus lining in AIC doesn't prevent access of the agent to cells and doesn't cause a delay of its effect but might act as a scavenger, reducing an amount of active maltopyranoside, available for acting upon cells. In accordance with this observation the permeability to EPO in the presence of MP C16 was enhanced far more efficiently in LCC. Differences in P_{app} values between the AIC and LCC model were more evident by
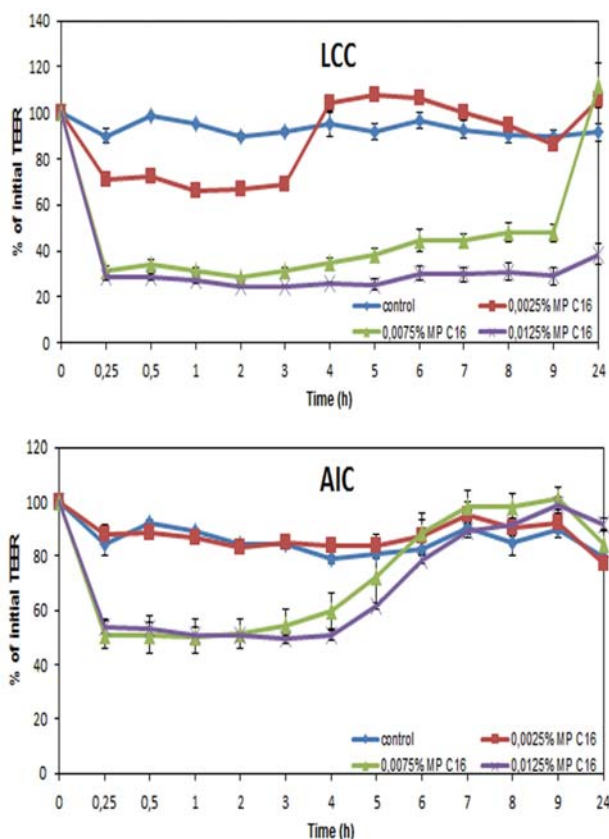

Figure 5. Effect of MP C16 on TEER values of Calu-3 epithelium models. TEER values were measured on LCC (a) and AIC (b) Calu-3 models during 3-hour MP C16 treatment and during 24 hours regeneration period after its removal. Data are expressed as percentage from the initial value and represent an average ±SD (n = 3).

increasing concentration of MP C16. We measured up to 60 fold increase in permeability under regenerative concentration (0.0075%) and up to 166 fold increase after addition of 0.0125% MP C16 on the LCC model. On the other hand, in the presence of the higher concentration on the AIC model, P_{app} for EPO was increased only 2.8 fold (Table 3). Changes in TEER were smaller and fully regenerative. Higher EPO permeability enhancement on the LCC model is partially a consequence of lower permeability potential before affecting it with MP C16.

The cultures of Calu-3 on the permeable supports that underwent MP C16 treatment were also inspected under the light microscope. Visible changes in the cell layer were observed after exposure in LCC. At 0.0075% of MP C16 cell borders became distinctive again alike to young cultures and missing cells were spotted within the layer at the highest treatment concentration (0.0125%). However, removal of MP C16 enabled complete regeneration of cell monolayer in the next 24 hours in case of lower concentrations whereas 0.0125% of MP C16 caused irreversible damage to cells in LCC model. In AIC no such changes were observed after a treatment with the same concentrations of MP C16 (Fig 6).

Table 3. Permeability of Calu-3 model to EPO when cultured in LCC and AIC conditions and when affected with indicated concentrations of MP C16. TEER is presented as a percentage of negative control. Permeability to EPO is presented as P_{app} values. Values are an average of two independent experiments or an average of three repeats within experiment. Factors represent the fold increase above the permeability of control treatment (0% MP C16).

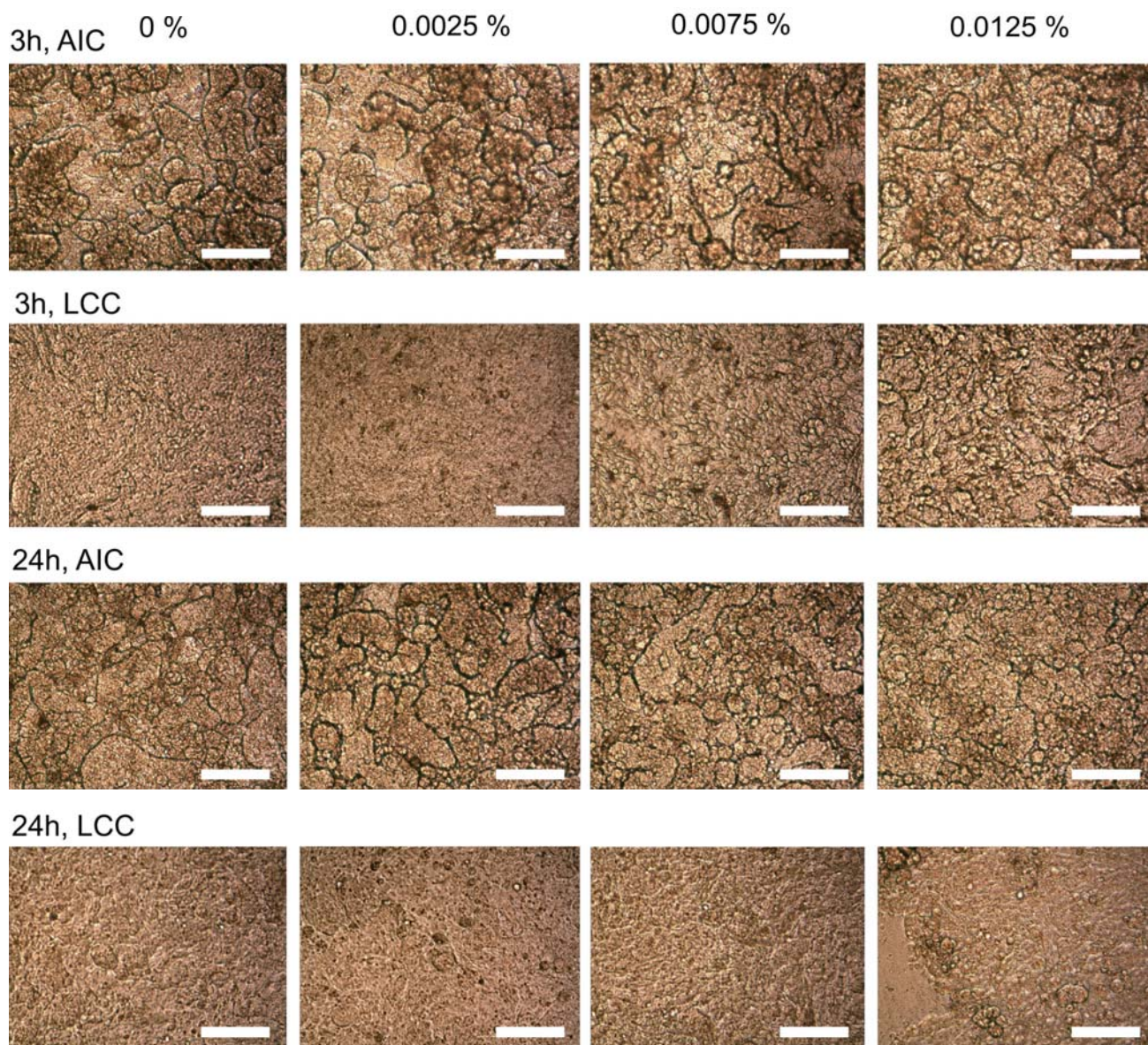| Culturing condition	Concentartion of MP-C16 (% w/v)	TEER (%)	Regeneration of TEER	P_{app} EPO (cm/s)	Factor
LCC	0			1.5×10^{-9}	1.0
LCC	0.0025	82 ± 18	Yes	6.7×10^{-9}	4.4
LCC	0.0075	35 ± 4	Yes	9.1×10^{-8}	59.3
LCC	0.0125	27 ± 5	No	2.6×10^{-7}	166.1
AIC	0			4.6×10^{-9}	1.0
AIC	0.0025	81 ± 5	Yes	7.3×10^{-9}	1.6
AIC	0.0075	57 ± 4	Yes	8.5×10^{-9}	1.9
AIC	0.0125	50 ± 1	Yes	1.3×10^{-8}	2.8

Figure 6. The change in appearance of MP C16 treated Calu-3 models under the light microscope. Cells grown in AIC and LCC conditions were treated with 0%, 0.0025%, 0.0075% and 0.0125% of MP C16 and obsereved 3 and 24 hours after treatment. Pronounced cell borders and cell detachment were detected only in treated LCC. Note presence of mucus in AIC. White bars represent 100 μm.

The toxicity of the MP C16 at the same concentrations was further measured by MTT assay on microtiter plates where Calu-3 cells are not cultured in a way to resemble either of the models (Fig 7). The cell viability signal was detected after 3 hour of treatment and after 24 hour regeneration period in fresh medium. After treatment with 0.0125% MP C16, cell viability was reduced to only 50% of the control treatment showing a clear toxic effect. On the other hand, in the case of 0.0075% MP C16 cell viability was evidently more preserved. However, we have measured very high variability among different experiments for this concentration of MP C16. Certain regeneration was observed for both concentrations in the next 24 hours. The excess in toxicity measured in microtiter plate over the one assumed from appearances of cells on inserts may be in part related to MP C16 dilution in the later due to transition of MP C16 to basal compartment.
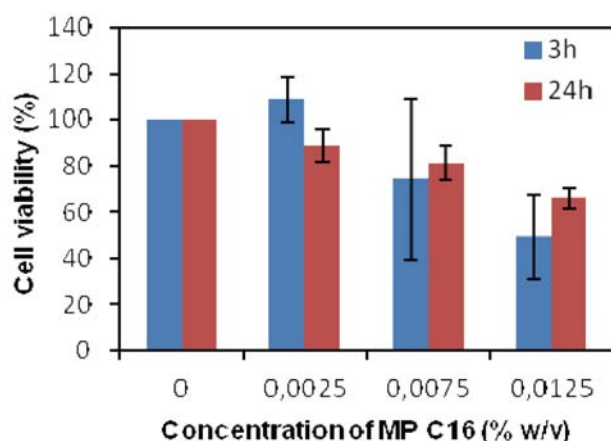

Figure 7. MP C16 cytotoxicity on Calu-3 cells. Cell viability as MTT signal derived from cultures after 3-hour MP C16 treatment and after 24-hour regeneration period. Results are expressed as a percentage of the negative control (0% MP C16) and represent averages ±SEM of three independent experiments. Viability signal was significantly reduced at highest concentration of MP C16.

Our data therefore suggest MP C16 to be a potent modulator of epithelial model permeability to proteins, being in concordance with reported *in vivo* study about its enhancing effects on the nasal absorption of insulin.[21] However, these results demonstrate that a very narrow range between effective and toxic concentrations for this permeability enhancer makes a safety of its application *in vivo* quite questionable.

Results presented in this paper together show through various means that AIC form of Calu-3 respiratory epithelium model is not simply mucus covered LCC model and that changes in cell layer along with contribution of mucus form a model of distinct properties. Both LCC and AIC models proved their usefulness for protein permeability testing. While the AIC model enables eva-

luation of mucus contribution to overall epithelial barrier function, the LCC model was found more suitable for assessing effects of the permeability enhancer on cellular barrier itself. Moreover, parallel application of both Calu-3 models could be useful for studying a mechanism of transport across the respiratory epithelium where the AIC model with thicker and mucus covered cell layer represents less permeable barrier for transcellularly transported compounds. On the other hand the LCC model with, higher TEER values exhibits lower permeability for paracellular permeators.

4. Conclusions

Calu-3 cell line based AIC and LCC respiratory epithelium models differ in their morphological and physiological properties. The two models exhibit different permeability properties regarding polarity and size of transported molecules and respond differently to MP C16, the detergent accelerator of permeability. While the LCC model enables quantification of the MP C16 related cell barrier modulator effect, the AIC model demonstrates mucus related diminishing effect on transport acceleration. According to this both models could contribute to characterization of transepithelial transport.

5. Acknowledgements

Abbreviations: TEER, transepithelial electrical resistance; P_{app}, apparent permeability coefficient; MP C16, N-hexadecyl-β-D-maltopyranoside; AIC, air interfaced culture; LCC, liquid covered culture; CMC, critical micelle concentration

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Povzetek

Obsežna površina dihalnega epitelija z blagimi razmerami na svoji površini je privlačna možnost, ko razmišljamo o prekosluznični dostavi proteinskih zdravil. Mukusni in celični sloj dihalnega epitelija lahko v pogojih *in vitro* primerljivo oblikujemo s celično kulturo Calu-3. Med razvojem sloja Calu-3 pod plastjo gojišča ali med izpostavitvijo zraku in tvorbo mukusne plasti smo sledili morfološkim spremembam in prehodnosti kultur. Poleg morfoloških razlik, sta se kulturi razlikovali tudi v električni upornosti in prehodnosti za proteinske molekule. Pospešeno prehodnost kultur za proteine smo preučevali ob prisotnosti modulatorja permeabilnosti MP C16. Spremembe električne upornosti celičnega sloja so bile hitre pri obeh načinih gojenja, kar kaže na lahko dostopnost MP C16 do celic, čeprav je bil celokupen učinek na prehodnost za proteine mnogo manjši pri kulturah z mukusom. Razlike v lastnostih dveh kultur Calu-3 omogočajo lažje razumevanje sluznične prehodnosti za proteine, poti njihovega prehoda in delovanja MP C16.