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Research paper

RPMI 2650 epithelial model and three-dimensional reconstructed human nasal mucosa as in vitro models for nasal permeation studies

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ABSTRACT

The purpose of this study was to investigate the human nasal epithelial cell line RPMI 2650 regarding its usefulness as in vitro model for drug permeation studies. Particularly, the influence of the air–liquid interface in culture and coculture with human nasal fibroblasts (HNF) on the differentiation and permeation barrier properties of the cell layer was examined. In addition to a non-contact coculture, we developed a three-dimensional construct of the human nasal mucosa composed of a collagen matrix with embedded HNF, covered by a RPMI 2650 epithelial cell layer.

Microscopic examination as well as measurement of the transepithelial electrical resistance and permeation experiments showed the importance of cultivation at the air–liquid interface. Permeation studies were performed using a paracellular marker (sodium fluorescein), a transcellular marker (propranolol-HCl) and a model substance with high molecular weight (FITC-dextran, MW 4000). The epithelial model showed an organotypic permeation barrier for paracellular, transcellular and high MW permeation. Three-dimensional reconstructed human nasal mucosa showed four- to fivefold higher permeation coefficients. Regardless of the limits of these models, both offer promise to evaluate passive drug permeation through the nasal mucosa.

1. Introduction

The nasal mucosa has a number of advantages as a delivery route for drugs with systemic action and has therefore become a promising alternative for drug administration. It shows advantages over other applications due to its leaky epithelium compared to intestinal epithelium or stratum corneum, extensive vascular supply and direct blood transportation into the systemic circulation, relatively large surface due to numerous microvilli and avoidance of first-pass metabolism. Other advantages of nasal administration include the possibility of self-administration and the avoidance of injections, which both can increase patient compliance [1]. Over the last decades, the nasal route has become increasingly important for drug application. From 1998 to 2008, the number of nasally applied substances with systemic action on the US market increased from five to nine, the Rote Liste® 2008 counts nine products as well on the german market [2–4]. New and established substances include small molecules such as triptanes (MW 300) and large peptide molecules such as calcitonin (MW 3421). Numerous approaches have been made to develop a nasal drug delivery system for insulin (MW ca. 5800). Increasing permeation and absorption of these large molecules have been a demanding task for researchers [5,6].

Introducing a new substance for nasal administration requires biopharmaceutical studies such as bioavailability studies. Before in vivo preclinical testing, it is reasonable to screen possible drug candidates with respect to their permeability through the nasal mucosa using an appropriate in vitro model. To study drug transport and permeation through the nasal mucosa, different in vitro models of the nasal mucosa have been developed [7–9]. The general aim is to develop a model with organotypic properties, particularly permeability. Three basic approaches are possible: The use of excised tissue (human, bovine, porcine, rabbit, etc.), the use of primary or serially cultured human nasal epithelial cells (HNE) and the use of immortalized epithelial cells. These models can be compared by evaluating the transepithelial electrical resistance (TEER) and permeation coefficients of different marker substances.

The first approach is the use of excised tissue. Tissues of animal origin can easily be obtained from slaughterhouses. Problems include differences between the species in enzyme activities or in cell type distribution. Rabbit, bovine and porcine mucosa have been widely used for permeation studies and examination of permeation enhancers. Also, the correlation with human nasal absorption has been satisfactorily confirmed [10–16].

The second possibility is the use of primary or serially cultured human nasal epithelial (HNE) cells. Problems include the

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dependancy of primary cell culture models on repeated sampling of cells, the complex isolation, limited lifespan, questionable reproducibility due to inter and intraindividual differences between the donors and possible selective isolation and cultivation of tight junction forming cells. Currently available in vitro models have been discussed by Kim [17].

The third approach is to use an immortalized cell line with the advantages of genetic homogeneity and reproducibility of results. One strategy is to use the commercially available tissue model EpiAirway™, which is a model of respiratory tract tissue. It consists of normal human tracheal or bronchial epithelial cells and has been previously used for in vitro tests of nasal bioavailability [18–20]. The EpiAirway™ has been shown to have a pseudostratified ciliated epithelium with TEER values of ca. 550 $\Omega\,cm^2$ [21]. This closely resembles the conditions in the tracheobronchial epithelium, while the nasal respiratory epithelium has been reported to have much lower TEER values [11,22].

In this study, we wanted to examine the commercially available human nasal epithelial cell line RPMI 2650. This cell line was isolated in 1962 from a squamous cell carcinoma of the nasal septum [23]. So far, RPMI 2650 has mostly been described as unsuitable for permeation studies due to several reasons: RPMI 2650 cells have not shown differentiation into goblet cells or ciliated cells and do not express tight junctions, they form clusters with free spaces in between, instead of growing to confluence [24,25]. In contrast, Peter postulated RPMI 2650 cells may be a meaningful tool for metabolism studies and granted them some potential for permeation studies [26]. Other researchers have used RPMI 2650 monolayers for permeation studies with mucoadhesive microspheres [27]. Cells in submersed culture formed a permeation barrier for FITC-labeled dextran (MW 4000; FD-4), and differences between the formulations were clearly visible, however, a comparison with nasal mucosa was not provided.

Human nasal epithelium is not usually covered by a liquid layer like submerse-cultured cell monolayers. A recent study by Bai et al. [28] postulates that RPMI 2650 cells are able to form a confluent cell layer and develop sufficient transepithelial electrical resistance, as well as an appropriate permeation barrier, when cultured at the air–liquid interface [28]. Under these culture conditions, the cells also seemed to express tight junction proteins. Furthermore, this method has been shown to induce the differentiation of human nasal epithelial cells in primary and serial cultures better than in liquid-covered cultures [29–31].

Based on these findings, the goal of our study was to further evaluate the culture conditions necessary to optimize barrier properties. In particular, this study observed the influence of human nasal fibroblasts on the differentiation and barrier properties of the cell layer. This required establishing a method to isolate and cultivate human nasal fibroblasts. Contact and non-contact coculture of epithelial cells and human nasal fibroblasts (HNF) should be examined. Both types of cocultivation of epithelial cells and fibroblasts have been shown to induce differentiation of the epithelial cells. A model of airway epithelial cells cultured on a layer of human nasal or bronchial fibroblasts has been described to grow tighter, differentiate into a pseudostratified ciliated epithelium, generate a measurable TEER and to be able to be subcultivated to achieve much higher cell numbers from one isolation process. A communication between fibroblasts and epithelial cells generated by soluble and diffusible paracrine factors like keratinocyte growth factor (KGF), epidermal growth factor (EGF) or other cytokines leading to better differentiation has often been reported for different biological barriers. Some authors find an improvement already by adding fibroblast-conditioned medium, others describe the necessity of direct contact of the different cell types [32–40]. Therefore, in addition to the non-contact coculture, we developed a three-dimensional construct of the human nasal mucosa composed of a collagen matrix with embedded HNF covered by a RPMI 2650 epithelial cell layer. Furthermore, we tested a variety of media supplements regarding their influence on the barrier properties of the permeation barrier. Serum has been described to have a negative (opening) effect on tight junctions in retinal epithelial cells [41]. Therefore, we also tested the influence of two different serum concentrations on the barrier properties of the cell layer.

The focus of the permeation studies was on the passive permeation through the barrier via the paracellular and transcellular pathways, in addition to a model substance of high molecular weight. As a quick method to examine barrier integrity, transepithelial electrical resistance (TEER) was also observed.

2. Materials and methods

2.1. Materials

MEM Earle medium, DME medium, fetal calf serum (FCS), L-glutamine and non-essential amino acids (NEAA) were obtained from Biochrom AG (Berlin, Germany). Two percent EDTA solution was obtained from MP (Heidelberg, Germany). Penicillin plus streptomycin solution was purchased from PAA Laboratories GmbH (Pasching, Austria). Trypsin solution was obtained from Gibco® Invitrogen GmbH (Karlsruhe, Germany). Collagen type I was self-extracted from rat tails according to the method of Bell [42]. Petri dishes, Transwell™ cell culture assembly with filter inserts, culture flasks and plastic well plates were purchased from Corning Life Sciences (Omnilab, Bremen, Germany). EVOM™ Epithelial Voltohmmeter, Endohm chambers and STX2 electrodes were obtained from WPI (Berlin, Germany). Sodium fluorescein, propranolol-HCl, FD-4, retinoic acid, dimethyl sulfoxide (DMSO) and cholera toxin were purchased from Sigma (Seelze, Germany). Methanol was obtained from Fisher Scientific (Loughborough, UK), sodium dihydrogen phosphate from Merck (Darmstadt, Germany) and triethylamine from Fluka (Buchs, Germany). Krebs–Ringer-Buffer (pH 7.4, 275 mosmol) consisted of sodium chloride (116.4 mM), potassium chloride (5.4 mM), sodium dihydrogen phosphate (1 mM), sodium bicarbonate (25 mM), HEPES (15 mM), D-glucose monohydrate (5.6 mM), magnesium sulfate heptahydrate (0.8 mM), calcium chloride dihydrate (1.8 mM) (Roth, Karlsruhe, Germany) and double-distilled water. All other chemicals used were analytical grade.

2.2. Tissue

Normal human nasal tissue specimens were obtained from turbinectomy surgeries in accordance with ethical regulations. Specimens were put into a physiologic sodium chloride solution immediately after removal. The specimens were cooled and used for experiments within 1 h after removal. Measurement of transepithelial electrical resistance as well as permeation experiments was carried out according to the proceeding description for reconstructed human nasal mucosa (Section 2.5.2).

2.3. Cell culture

2.3.1. RPMI 2650

The cell line RPMI 2650 was isolated in 1962 from a squamous cell carcinoma of the nasal septum [23]. Cells were purchased from the German Collection of Microorganisms and Cell Cultures (ACC 287, DSMZ, Braunschweig, Germany) and used in passages 5–35. The cells were maintained in a culture medium containing MEM Earle, 10% fetal calf serum (FCS), 1% L-glutamine, 1% NEAA and 1% antibiotics. At approximately 80% confluence (Fig. 1A), the cells were washed with EDTA solution and detached from the surface by

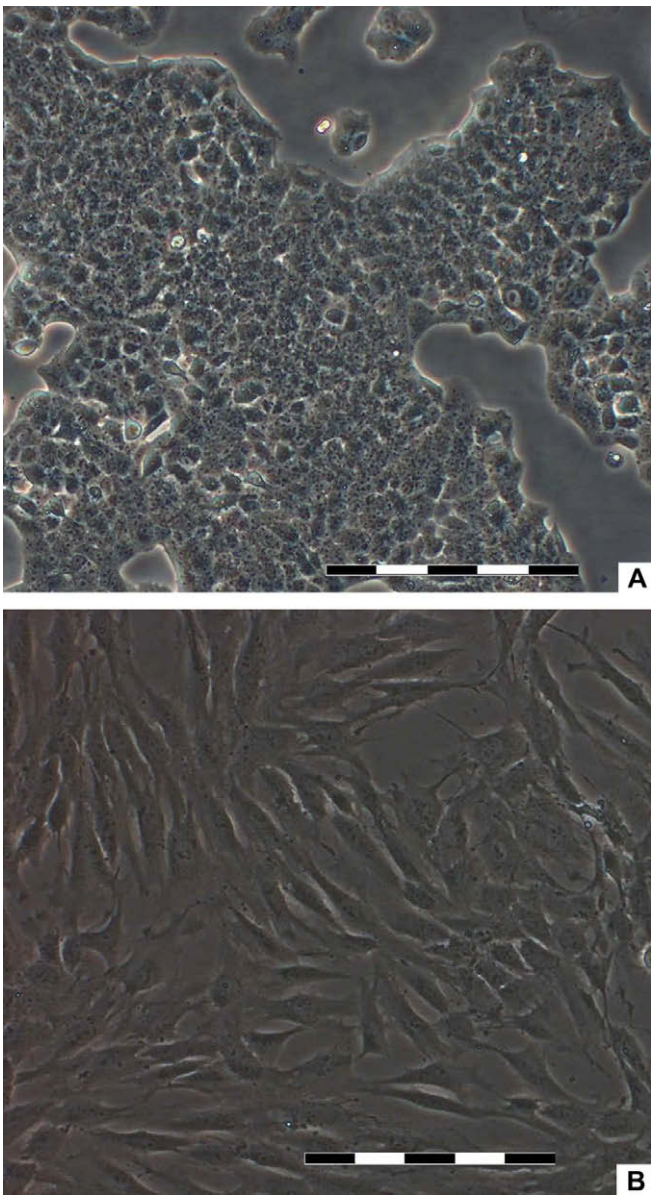

Fig. 1. (A) Eighty percent confluent RPMI 2650 cells grown in a 25 cm^2 plastic flask as viewed under a light microscope (bar repr. 200 μm). (B) HNF in passage 1 grown in a 25 cm^2 plastic flask as viewed under a light microscope (bar repr. 200 μm).

trypsinization. After resuspending and counting, cells were seeded at a density of 40,000 cells/cm^2 in a new culture flask. Cells used for experiments were mycoplasma-free, as determined by MycoAlert® (Lonza, Rockland, USA).

2.3.2. Human nasal fibroblasts (HNF)

HNF were isolated using specimens of normal human nasal mucosa obtained after turbinectomy. After washing the specimen with Krebs–Ringer-Buffer, the epithelial cell layer was removed by scraping, and the underlying connective tissue was cut into pieces of 1–2 mm^2. These pieces were attached to the surface of a petri dish and were allowed to incubate in cell culture medium (DMEM supplemented with 1% L-glutamine, 1% antibiotics and 10% fetal calf serum) for 10–14 days. Fibroblasts grew out of the tissue after 5–10 days and could be harvested by trypsinization. From passage 1 onward, the fibroblasts showed a spindle-like appearance (Fig. 1B), and therefore fibroblast phenotype was morphologically confirmed.

Cell growth and collagen-contraction experiments showed the cells remained viable up to passage 3; thereafter, cell multiplication decreased while the contracting activity remained stable up to passage 4. Hence, the HNF cells were used up to passage 3.

2.4. RPMI 2650 epithelial model

For the standard RPMI 2650 epithelial model for permeation experiments, RPMI 2650 cells were seeded onto Transwell™ filter inserts (3 μm pore size, polycarbonate) at a density of 200,000 cells/1.13 cm^2. The cultures were maintained in a liquid-covered culture for 8 days, and medium was replaced every 2–3 days. After 8 days, the inserts were either lifted to an air–liquid interface culture, or kept in liquid-covered culture. After two more weeks (days 21–23), the RPMI models could be used for experiments. TEER measurements and permeation experiments were conducted in triplicate.

Variations of this standard model were also tested: The serum concentration was lowered down to 2%, the filter insert was coated with collagen (rat tail acid extracted type-I-collagen) before seeding and a non-contact coculture of HNF and RPMI was tested. Collagen coating was conducted one day before seeding. Hundred microlitre of a solution of rat-tail collagen (1.5 mg/ml collagen in 0.025% acetic acid, 50% V/V ethanol) was cast onto the filter insert and dried overnight. Non-contact coculture of HNF and RPMI 2650 was arranged by seeding 20,000 HNF on the bottom of 12-well plates on day 1 (equal to 5260 cells/cm^2), on day 2, RPMI 2650 were seeded as described, onto Transwell™ filter inserts, which were inserted into the plates.

2.4.1. TEER measurements

Transepithelial electrical resistance (TEER) was measured using an EVOM with additional Endohm chamber. Transwell™ inserts were allowed to reach room temperature and then measured in a chamber containing the respective culture medium. For examination of the influence of different media supplements on the barrier properties of RPMI 2650 cells, the standard epithelial model was cultivated using different media. Influence of media supplements as well as liquid-covered culture (LCC) or air–liquid interface (ALI) cultivation was examined. Media composition is listed in Table 1. Values of blank filters were subtracted, and corrected values were calculated corresponding to the surface area.

2.4.2. Permeation experiments

The following variations of cultivation were tested: collagen treatment of the surface, cocultivation with HNF, high or low serum concentration and LCC or ALI culture. The first approach was to test the passive permeation through the barrier. As a marker substance for paracellular permeation sodium fluorescein (donor concentration 25 μg/ml) was chosen, for transcellular permeation propranolol-HCl (1 mg/ml), and as a marker with high molecular weight FITC-dextran 4000 (FD-4, 500 μg/ml) was tested. All marker substances were tested as follows: the RPMI 2650 epithelial model

Table 1
Media composition used in TEER experiments.

Basic medium	Additional supplement
MEM Earle	**A** No supplement
+1% L-glutamine	**B** +Cholera toxin 10 ng/ml
+1% NEAA	**C** +Retinoic acid 10 ng/ml
+10% FCS	**D** +DMSO 5%
MEM Earle	**E** No supplement
+1% L-glutamine	**F** +Cholera toxin 10 ng/ml
+1% NEAA	**G** +Retinoic acid 10 ng/ml
+2% FCS	**H** +DMSO 5%

was prepared and cultivated, as described, on Transwell™ filter inserts. The substance was dissolved in KRB (Krebs–Ringer-Buffer) being the donor solution. Cultivated cells were washed once with prewarmed KRB and equilibrated for 1 h in KRB at 37 °C. Then, the filter inserts were transferred to a new plate with 1.5 ml prewarmed acceptor buffer (KRB). On the apical side, 0.5 ml of the donor was cast and time measurement started. Samples of 100 μl (except for propranolol-HCl, sample volume 500 μl) were taken from the acceptor at fixed time intervals and replaced with prewarmed KRB. During the experiment, the plates were shaken horizontally with 200 U/min at 37 °C. There was no considerable change in TEER during the experiments.

Propranolol was quantitatively analyzed by HPLC using Waters 515 HPLC Pump, Waters 717 plus Autosampler, Waters 486 tunable Absorbance Detector and Clarity software (mobile phase: methanol/phosphate buffer pH6/triethylamine 114:45:0.2, stationary phase: LiChrospher 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany), detection wavelength 227 nm). The calibration correlation coefficient was 0.9999. Sodium fluorescein and FD-4 were analyzed by fluorescence spectroscopy using a Tecan Genios fluorescence plate reader and Magellan software (excitation wavelength 485 nm, emission wavelength 535 nm).

Samples were analyzed, and the permeation coefficient (P_{app}) was calculated according Eq. (1):

$$P_{app} = \frac{dQ}{dt \cdot c_0 \cdot A}, \quad (1)$$

where dQ/dt is the flux (μg/s) of the respective substance across the barrier, c_0 is the initial donor concentration (μg/ml) and A is the surface area (cm^2).

2.5. Reconstructed human nasal mucosa (constructs)

To advance the RPMI epithelial model, a contact coculture of RPMI and HNF in a three-dimensional construct was developed. Reconstructed organotypic three-dimensional models of permeation barriers have been shown to be able to mimic in vivo conditions better than epithelial models. To make the model as organotypic as possible, HNF and RPMI 2650 should be cocultivated not only in the same culture well, but also in direct contact with each other. A collagen matrix containing fibroblasts was used as a growth support for the epithelial cells. Constructs including human nasal fibroblasts (HNF) and RPMI 2650 were built up step-by-step in Transwell™ cell culture inserts (24 mm diameter, pore size 3 μm). The cultures were maintained at 37 °C and 5% CO_2 in a humidified atmosphere. HNF were used from the second and third passage, RPMI 2650 were used in passages 5–13. For a six-well Transwell™ plate, HNF were suspended in specific quantities in culture medium. Collagen type I (8 ml at 4.8 mg/ml) isolated from rat tails in acetic acid (0.05%) was mixed with 11 ml acetic acid (0.05%) and 5.6 ml culture medium containing $NaHCO_3$ (8.9 mg/ml). HNF suspension (2 ml) were added, and 4 ml of the HNF containing collagen solution were then cast into each well of six-well Transwell™ inserts and allowed to thicken. The thickened gels were detached from the underlying filter on day 1 after seeding and fed in liquid-covered culture (LCC) with HNF-medium. The amount and viability of the HNF was clearly visible by the extent of gel contraction, which started on day 2 after seeding. On day 7, 100,000, 200,000, or 400,000 RPMI 2650 epithelial cells were suspended in 50–70 μl of medium and seeded onto the collagen–HNF-matrix. They were allowed to attach on the surface for 2 h and then fed with RPMI 2650-standard medium. These constructs were cultivated for one week in LCC and then lifted to air–liquid interface (ALI), where they were cultivated from the basal side with 3-4 ml medium every 2–3 days. After two more weeks (day 28 after HNF-seeding), the constructs were used for permeation or histologic embedding. TEER measurements and permeation experiments were conducted in triplicate.

2.5.1. TEER measurements

Constructs were mounted in modified Ussing permeation chambers and examined after 1 h of equilibration in Krebs–Ringer-buffer (KRB) at 37 °C with STX electrodes and EVOM. Values were calculated corresponding to the surface area.

2.5.2. Permeation experiments

Constructs were permeated in modified Ussing permeation chambers with 2 ml donor and acceptor volume. They were equilibrated 1 h in KRB at 37 °C, before KRB was replaced by fresh KRB as acceptor or a solution of the marker substance in KRB as donor. Donor concentrations were as described for the epithelial model. Sample volume was 200 μl (except for propranolol-HCl, sample volume 600 μl). There was no considerable change in TEER during the experiments.

2.6. Light microscopy

Specimens were fixed in formaldehyde solution overnight, dehydrated by increasing ethanol concentration and embedded in Technovit® 7100 from Heraeus Kulzer (Wehrheim, Germany). 4–5 μm sections were prepared by means of a Microm H355 S microtom (Walldorf, Germany), stained in hematoxylin eosin and examined with a Olympus IX50 photomicroscope using Cell^F software.

2.7. Statistical analysis

Where appropriate, analysis of variance and Student's t-test were employed to determine statistical significance of in vitro experiments ($p < 0.05$).

3. Results and discussion

3.1. Light microscopy

3.1.1. RPMI 2650 epithelial model

RPMI 2650 cells were cultivated on polycarbonate Transwell™ filter inserts (pore size 3 μm) and examined during their cultivation with respect to the free area on the filter. Cells in LCC grew in clusters, as described before [25], and did not reach confluence. ALI-cultured cells showed a different growth pattern, they began to overgrow the free filter area after being set to ALI. Fig. 2 shows cross-sections of RPMI 2650 epithelial models either grown in LCC or grown in ALI. It was clearly visible that LCC developed a leaky and flat cell multilayer, while ALI cultures developed a relatively tight, thick, homogenous cell multilayer. The multilayer does not resemble a respiratory epithelium for lack of a ciliated pseudostratified columnar epithelium. It consists of 10–15 layers of mostly cuboidal shaped cells, which show some kind of differentiation from cells growing directly on the filter up to the cells at the surface in contact with the air.

3.1.2. Reconstructed human nasal mucosa

Micrographs of the constructs showed a collagen matrix with embedded spindle-like fibroblasts covered by a multilayer of RPMI 2650 epithelial cells (Fig. 3). The structure of the multilayer resembles the structure of the Transwell™-cultured mutlilayers with ALI.

Primary or serially cultured HNE form a ciliated epithelium with tight junction complexes. Also, goblet cells are present, and mucin secretion has been described [43,44]. The EpiAirway™ tissue is described as a ciliated, tight junction forming pseudostratified

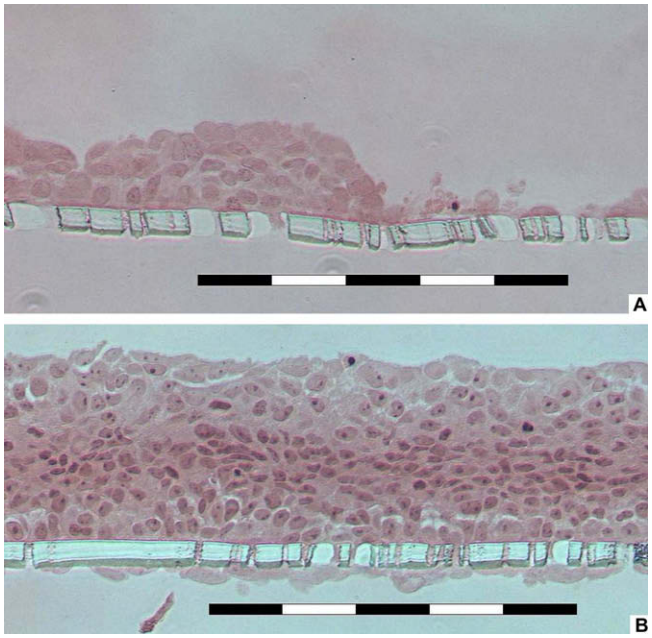

Fig. 2. HE-stained cross-sections of RPMI 2650 epithelial model cultivated on Transwell™ inserts after 3 weeks, A: LCC, B: ALI (bar repr. 200 μm).

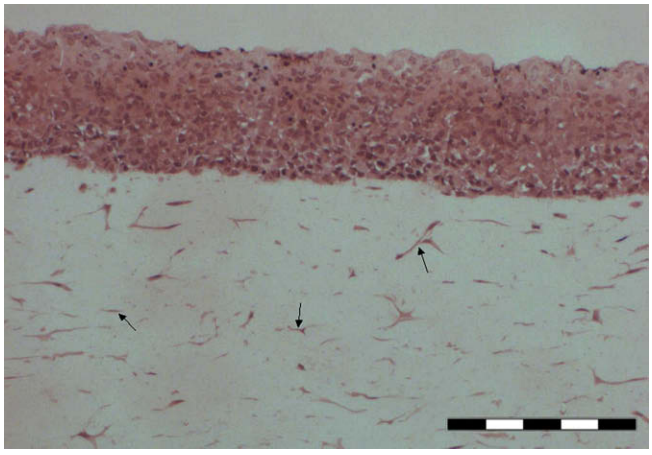

Fig. 3. HE-stained cross-section of three-dimensional reconstructed human nasal mucosa consisting of 100,000 HNF and 200,000 RPMI 2650, 4 weeks after HNF-seeding. The arrows indicate fibroblasts (bar repr. 200 μm).

epithelium resembling human bronchial epithelium. It consists of three- to four-cell layers when cultured at ALI [21]. Both our epithelial model and reconstructed nasal mucosa do not show a pseudostratified or ciliated morphology as in vivo. However, cell differentiation, when cultivated under ALI conditions, is visible, and a tight multilayer is formed (Fig. 2).

3.2. TEER measurements

3.2.1. RPMI 2650 epithelial model

The first approach to test the barrier function of cultured RPMI 2650 cells was the evaluation of different culture media and conditions, and their effect on transepithelial electrical resistance (TEER). TEER values are a good indication of the integrity of an epithelial barrier. As shown in Fig. 4, on day 23 after seeding on polycarbonate filters, no significant difference ($p < 0.05$) between different media compositions was detectable. Cells grown in a submersed culture showed slightly higher TEER when supplied with 10% serum, compared to 2%. However, none of the supplements was able to increase the TEER significantly, compared to the basal medium (10.5 Ω cm^2). TEER of epithelial models was strongly dependent on cultivation at an air–liquid interface. Only multilayers under ALI conditions were able to reach confluence, while multilayers in LCC grew in clusters with free spaces in between (Fig. 2). ALI cultures reached TEER values of 75 Ω cm^2 (basal medium with 10% serum) down to 58 Ω cm^2 (basal medium with 2% serum). Neither HNF coculture, nor collagen treatment changed TEER values (data not shown). The highest TEER value of an RPMI epithelial model found in this study was 75 Ω cm^2. TEER values of ALI cultures were significantly ($p < 0.05$) higher than the TEER of LCC.

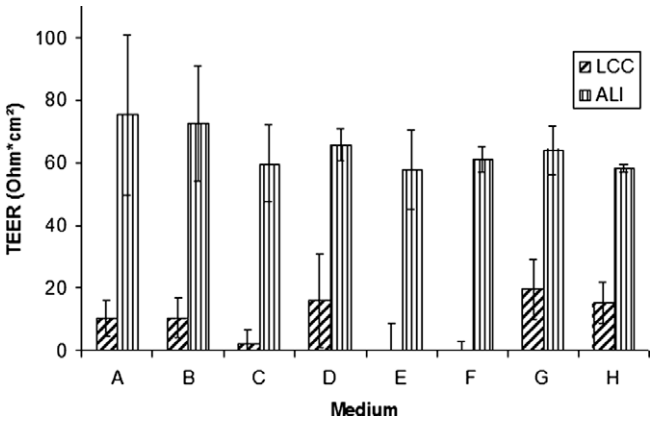

Fig. 4. The TEER of RPMI 2650 epithelial models cultivated on Transwell™ inserts on day 23 after seeding. The dependence on culture medium (see Table 1) and ALI, $n = 4$, mean ± SD.

3.2.2. Reconstructed human nasal mucosa

TEER values of constructs were slightly, but not significantly, higher, but in the same range, as those detected for RPMI multilayers (Fig. 5). Higher numbers of RPMI 2650 cells resulted in slightly, but not significantly, higher TEER. In addition, higher numbers of HNF had no significant influence on TEER. Mean TEER values from 57 Ω cm^2 (25,000 HNF and 100,000 RPMI) up to 78 Ω cm^2 (50,000 HNF and 400,000 RPMI) were detected.

These results must be related with TEER values of nasal mucosa in humans, as well as in other animals. We tested excised human and porcine nasal mucosa and found values of 90–180 Ω cm^2 (human) and 100–198 Ω cm^2 (porcine). Both inter and intraindividual

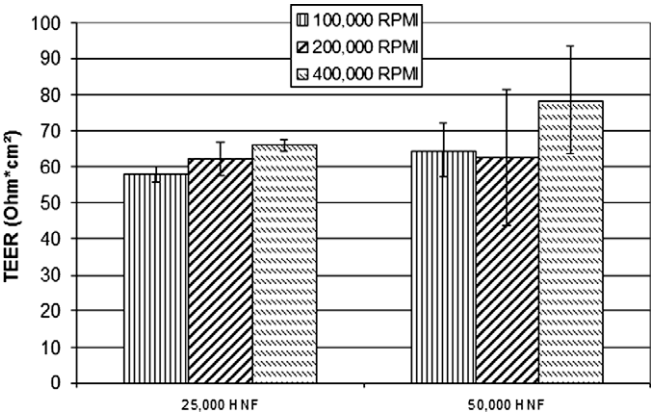

Fig. 5. The TEER of reconstructed human nasal mucosa before permeation on day 28 after seeding of HNF. The dependence on the number of HNF in the collagen matrix and on the number of RPMI epithelial cells, $n = 3$, mean ± SD.

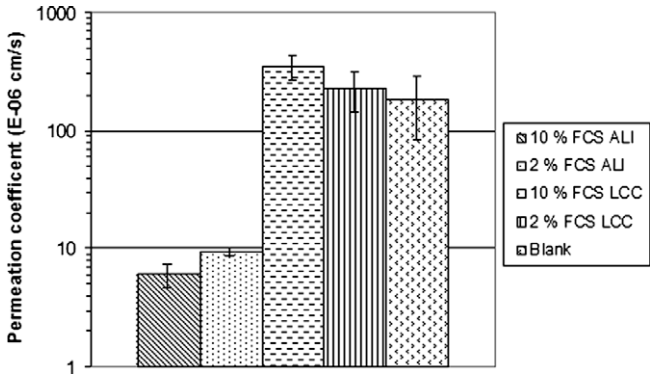

Fig. 6. Permeation coefficient (10^{-6} cm/s) of sodium fluorescein through RPMI 2650 epithelial models cultivated on Transwell™ inserts, 21 days after seeding, $n = 3$, mean ± SD.

variation were high. Reported values of porcine mucosa ranged from 30 to 250 Ω cm^2 [11]. Human mucosa ranged from 50 to 100 Ω cm^2 [22,45]. The RPMI epithelial model showed a slightly lower TEER, but it is close to the reported values of nasal mucosa. Data showed that the constructs seem to be fairly organotypic, regarding TEER. Reconsidering the reported values of primary cultures (200–3450 Ω cm^2) [29,43,44,46], we can conclude that the RPMI 2650 models are closer to the physiologic conditions regarding TEER. Furthermore, RPMI 2650 models seem to be more reproducible.

3.3. Permeation experiments

3.3.1. Sodium fluorescein

Sodium fluorescein was tested as a hydrophilic marker substance for paracellular permeation. The tightest barrier was achieved after three weeks of culture without collagen treatment or HNF cocultivation, in a 10% serum concentration and ALI after one week of LCC (standard cultivation). The permeation coefficient was 6.09×10^{-6} cm/s and was significantly lower than permeation coefficients of all other cultivations ($p < 0.01$), except HNF coculture due to the high standard deviation for this treatment (Fig. 6, Table 2). The difference between LCC and ALI culture is apparent. LCC shows a permeation coefficient of 3.49×10^{-4} cm/s, which is almost 60-fold higher and in the range of free diffusion through blank filter ($P_{app} = 1.86 \times 10^{-4}$ cm/s). These findings correlate with the microscopic examinations of confluence, which is not reached by LCC. The results confirm the ALI culture to be essential for the formation of a tight permeation barrier by RPMI 2650 cells.

Bai et al. found an increased expression of tight junctional structures and proteins when RPMI cells were kept at an air–liquid interface and reported a permeation coefficient of 5.07×10^{-6} cm/s for mannitol as a paracellular marker [28]. This epithelium was cultivated on polytetrafluoroethylene (PTFE) filters instead of polycarbonate filters. Although a different paracellular marker is used, this model seems to be slightly tighter, as TEER values of appr. 150 Ω cm^2 on PTFE filters compared to 75 Ω cm^2 on polycarbonate filters (both 3 μm pore size) confirm. However, if a PTFE growth surface supplies better possibilities for differentiation and formation of tight junctional structures, it has to be examined in a direct comparison.

The constructs presented three- to sixfold higher permeation coefficients than the epithelial model, reaching $1.84–3.59 \times 10^{-5}$ cm/s, depending on the number of RPMI 2650 cells and HNF (Table 3). Permeation barrier properties of the constructs correlated with TEER values. A slight decrease of the permeation coefficient with increasing number of epithelial cells is visible. The influence of the HNF can additionally be explained by the HNF-dependent contraction of the collagen matrix leading to slightly increased thickness.

Excised human nasal mucosa showed a mean permeation coefficient of 3.12×10^{-6} cm/s ± 1.99×10^{-6} cm/s, showing a wide interindividual variation. Permeation coefficients of isolated primary epithelial cells are reported as $0.045–0.191 \times 10^{-6}$ cm/s [46], which is about 15- till 70-fold tighter than mucosa. A difficulty with culturing cells from the nasal mucosa is that the mucosa consists of different cell types, which are not equally viable in primary culture. It has been described that in cultures of murine nasal epithelial cells, no goblet cells could be detected after three weeks of culture [47]. Therefore, after three weeks, the culture did not represent these cell types in the same proportion as in the in vivo epithelium. It has been reported that filled goblet cells lead to discontinuity of tight junctions [48–50]. This finding may suggest the existence of leaky areas in between the tight junction forming columnar epithelial cells. It is possible that due to selective isolation of tight junction forming cells in primary culture, these cultures result in tighter barriers than physiologic relations of cell types would. Based on this, one must consider if isolated nasal epithelial cells in primary culture will be able to mimic physiologic conditions, or if, by selective isolation and cultivation of tight junction forming cells, there will be a tighter epithelium than in vivo.

There was no significant difference between the RPMI 2650 epithelial model and excised mucosa, which correlates with the TEER values. The reconstructed human nasal mucosa seems to be leakier than excised tissue and the epithelial model, which should be noted when using it for paracellular permeation studies. One possible reason is the more complex handling of the constructs; the experimental set-up leads to mechanical shear stressing during the transfer into the permeation chambers. Improvement of the constructs could be achieved by increasing the solidity of the collagen matrix. Furthermore, a serum-free cultivation inhibits the contraction of the collagen matrix by the fibroblasts and could therefore lead to constructs that can be examined directly in the Transwell™ filter inserts.

3.3.2. Propranolol-HCl

Propranolol-HCl was chosen as a lipophilic marker substance for transcellular permeation. Usually, transcellular permeation is

Table 2
Permeation coefficients (10^{-6} cm/s) of sodium fluorescein through RPMI 2650 epithelial models, day 21 after seeding and excised human nasal mucosa, $n \geqslant 3$, mean ± SD.

	Standard cultivation	HNF coculture	Collagen coating	Blank filter
10% FCS ALI	6.09 ± 1.38	17.5 ± 16.5	14.2 ± 2.9	185.7 ± 101.6
10% FCS LCC	349 ± 78		42.7 ± 7.5	
2% FCS ALI	9.38 ± 0.6	11.27 ± 0.6	29.63 ± 5.3	
2% FCS LCC	226.55 ± 81.5		60.3 ± 14.7	
Excised human mucosa	3.12 ± 1.99			

Table 3
Permeation coefficients (10^{-5} cm/s) of sodium fluorescein, propranolol-HCl and FD-4 through reconstructed human nasal mucosa, day 28 after seeding, $n = 3$, mean ± SD.

	0 RPMI	100,000 RPMI	200,000 RPMI	400,000 RPMI
25,000 HNF sodium fluorescein	4.65 ± 0.07	3.59 ± 0.07	2.80 ± 0.35	2.77 ± 1.05
50,000 HNF sodium fluorescein	6.4 ± 2.26	3.57 ± 0.25	2.06 ± 0.67	1.84 ± 0.83
50,000 HNF Propranolol-HCl			1.32 ± 0.15	
50,000 HNF FD-4			0.97 ± 0.18	

Table 4
Permeation coefficients (10^{-5} cm/s) of propranolol-HCl through RPMI 2650 epithelial models, day 21 after seeding, $n = 3$, mean ± SD.

	Standard cultivation	HNF coculture	Collagen coating	Blank filter
10% FCS ALI	3.1 ± 0.12	3.36 ± 0.31	3.18 ± 0.27	12.9 ± 0.21
10% FCS LCC	9.62 ± 1.53	12.3 ± 0.95	5.52 ± 0.27	
2% FCS ALI	4.72 ± 0.67	4.67 ± 0.50	4.08 ± 0.53	
2% FCS LCC	6.26 ± 2.23	8.07 ± 2.63	5.38 ± 0.24	
Excised human mucosa	2.0 ± 0.8 [15]			

not as dependent on tight junction forming as paracellular permeation, and therefore, the transcellular permeation rates are usually higher. For the RPMI epithelial model, the tightest barrier was also achieved with the described standard cultivation ($P_{app} = 3.1 \times 10^{-5}$ cm/s). A significantly higher permeation coefficient (threefold) was detected in LCC, while other variations of cultivation had no significant influence on the permeation barrier properties (Table 4). Reconstructed human nasal mucosa showed a slower permeation of propranolol-HCl (1.32×10^{-5} cm/s) (Table 3).

Bai et al. reported $1.6 \pm 0.1 \times 10^{-5}$ cm/s for their RPMI 2650 model [28]. Reported values of permeation coefficients for excised human mucosa are in the range of $2.0 \pm 0.8 \times 10^{-5}$ cm/s [15]. Kubo et al. examined excised rabbit mucosa and found a P_{app} of 2.6×10^{-5} cm/s for propranolol-HCl, which is only slightly lower than P_{app} of the epithelial model, while the reconstructed nasal mucosa is even tighter [51]. Isolated epithelial cells have been described as slightly less permeable for transcellular markers. Lee et al. found a permeation coefficient of 1.65×10^{-5} cm/s for budesonide through HNE. Forbes et al. reported 1.59×10^{-5} cm/s for propranolol-HCl through bronchial epithelial cells [9,29].

Regarding this transcellular marker, both the RPMI epithelial model and the constructs show similar barrier properties compared to excised nasal mucosa.

3.3.3. FD-4

FITC-labeled dextran (FD-4) was chosen as a hydrophilic model substance with high molecular weight (MW 4000). As seen before, the tightest barrier was achieved with the standard RPMI epithelial model ($P_{app} = 2.48 \times 10^{-6}$ cm/s). All other cultivation methods resulted in significantly higher permeation coefficients ($p < 0.01$) (Table 5). Reconstructed human mucosa also showed about a fourfold higher permeation rate (9.7×10^{-6} cm/s). Possibly due to mechanical shear stressing, it seems to be four- to fivefold leakier for paracellular permeation than the epithelial model. Excised mucosa has been shown to have similar barrier properties for high molecular weight substances. Excised rabbit mucosa showed a P_{app} of 1.275×10^{-6} cm/s for FD 4400 [51], and this value has been confirmed by Nakamura et al. (1.20×10^{-6} cm/s) [13].

Reported values for isolated cells are clearly lower, analogous to the paracellular permeation of sodium fluorescein. Experiments with different human respiratory epithelial cell culture models of 16HBE14o-, CaLu-3- and human alveolar epithelial (hAEpC) cells showed permeation coefficients of 1.53, 0.006 and 0.0171×10^{-6} cm/s, respectively. [52–54]. Werner and Kissel tested HNE cells and found a permeation coefficient of 0.18×10^{-6} cm/s, which is almost sevenfold lower than those of excised nasal mucosa [43].

Table 5
Permeation coefficients (10^{-6} cm/s) of FD-4 through RPMI 2650 epithelial models, day 21 after seeding, $n = 3$, mean ± SD.

	Standard cultivation	Collagen coating	Blank filter
10% FCS ALI	2.48 ± 0.72	7.55 ± 0.46	53.0 ± 5.56
10% FCS LCC	56.5 ± 6.9	13.5 ± 1.23	
2% FCS ALI	3.77 ± 0.56	10.4 ± 0.86	
2% FCS LCC	44.1 ± 1.69	14.6 ± 2.0	

As mentioned before, large molecules like the peptide calcitonin are established as nasally applied dosage forms. It is therefore obligatory for any in vitro model for nasal permeation to form an organotypic barrier for large molecules. The RPMI 2650 epithelial model appears to meet this demand.

4. Conclusion

Up until now, most researchers working with RPMI 2650 cells postulated that this immortalized cell line was not suitable for permeation studies and preferred to work with primary cultures of nasal epithelial cells. Only few authors suggest that this cell line has some potential for permeation studies [26,28]. According to this assumption, we analyzed different culture conditions, especially coculture of human nasal fibroblasts, and their influence on barrier properties of RPMI 2650 cells.

Our findings suggest that it is possible to form a permeation model of the nasal mucosa with RPMI 2650 cells. Of major importance is the cultivation at air–liquid interface, which permits a confluent growth. A lack of organotypic differentiation is certainly a limit of the model, which is morphologically not wholly organotypic. However, the permeation coefficients of the RPMI epithelial model were in the same range as human nasal mucosa and only about a factor of 1–2 higher than the excised tissue. A non-contact coculture with human nasal fibroblasts did not lead to increased TEER or lower permeation coefficients. If it can influence the differentiation of RPMI 2650 cells regarding other properties such as expression of active transporters is still to be analyzed.

A reconstruction of nasal mucosa was possible using isolated human nasal fibroblasts in collagen matrix covered by RPMI 2650 epithelial cells. These constructs show a differentiated non-respiratory-like epithelium. They also show permeation barrier properties comparable to excised nasal mucosa, while paracellular permeation turned out to be four- to fivefold faster than in the epithelial model. A disadvantage compared to the RPMI epithelial model is the more complex handling of the constructs.

In conclusion, we achieved a permeation barrier for passive permeation of hydrophilic, lipophilic and large compounds, which resembles the barrier properties of human nasal mucosa. Compared to the described permeation models based on primary cultures, the RPMI 2650 models are closer to the physiologic barrier properties for passive permeation. Further investigation is to be focused on active transport in the nasal mucosa. Different transporters have been shown to be expressed in human nasal mucosa [55,56].

Keeping in mind the limits of the models, both the RPMI 2650 epithelial model on Transwell™ filter inserts and the three-dimensional reconstructed human nasal mucosa model are promising models to evaluate passive permeation of substances through the nasal mucosa. Components are commercially available, cultivation is easy and fast and the results are reproducible without great variability.

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