Development of a Human Nasal Epithelial Cell Culture Model and Its Suitability for Transport and Metabolism Studies Under in Vitro Conditions

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Received June 16, 1994; accepted November 16, 1994

A human nasal epithelial cell culture model has been adapted to observe transport and metabolism of drugs, e.g., peptides. Human nasal epithelial cells, isolated by protease treatment of human nasal conchae, grew to confluency after 6-8 days using DMEM supplemented with 1% nonessential amino acids, 1% glutamine, 10% FCS and 1% antibiotics. These cultures expressed microvilli and actively beating cilia as documented by light microscopy and scanning electron microscopy (SEM). Tight junctions were confirmed by dome formation and positive actin staining using FITC-labelled phalloidin. Preliminary transport studies, carried out with FITC-labelled Dextran (FD 4, MW 4400) and Sulforhodamine (SR 101, MW 607), demonstrated the intact barrier function of the cultured monolayer, grown on filter membranes. In addition, the cultured cells metabolized Leu-Enkephalin to Des-Tyr-Leu-Enkephalin demonstrating the presence of aminopeptidase, a naturally occurring enzyme in the human nasal mucosa.

KEY WORDS: human nasal epithelium; primary cell culture; differentation; drug metabolism; drug transport.

INTRODUCTION

The increasing availability of proteins and peptides for chronic systemic therapy is the main driving force for the search of safe, convenient and noninvasive methods of drug delivery. Administration of peptides via the nasal route is an interesting alternative to parenteral drug administration due to the painless application, the highly vascularized mucosa of the nose allowing relative rapid absorption kinetics and the avoidance of the liver first pass effect.

Nasal absorption of several hormones and peptides, e.g., insulin (1,2), estradiol (3), human growth hormone (4,5), vasopressin (6), octreotide (7) and enkephalin (8,9) has been investigated. Most of these studies were conducted using animal models such as rabbits (1,3,4), sheep (4) or an *in situ* rat model (10). Disadvantages of such whole animal models are attributed to the differences in anatomy of the nasal cavity compared to the human nose, the large number of animals needed, the large quantities of drugs required and the difficulty in interpreting results influenced by unknown factors within the animal.

For this reason the development of an in-vitro cell culture model system for airway epithelial cells is currently subject of intensive research. Airway cells were mainly obtained from hamster trachea (11), or human bronchus (12,13), trachea (14) or nose (15–18). There are different basic approaches to culture airway epithelial cells in-vitro. One method used cells derived from explant outgrowth cultures (12,14,19) and another method seeded enzymically dissociated cells directly on culture dishes (11,15–18). Cells were cultivated in serum containing media usually supplemented by other growth factors (12,17,18) or in serum-free hormone-supplemented media (13–16). Some investigators treated their support materials with collagen films (18,19), others preferred collagen gels (15,16).

In fact, well differentiated cell cultures were scarcely reported. The cells became squamous, secretory and ciliated cells disappeared (15,17). Neither transport nor metabolic studies under in-vitro culture conditions have been conducted in cell monolayers of human nasal epithelial cells.

We report here the cultivation of human nasal epithelial cells in primary culture. These cells were well differentiated, preserved their cilia and grew to a confluent monolayer. The development of tight junctions enabled transport studies of two model drugs. In addition, the metabolic activity of the cultured cells was assessed using a model peptide, known to be extensively degraded by epithelial enzymes.

MATERIALS AND METHODS

Cell Cultures

Nasal tissue pieces of 1–3 cm^2 surface area were obtained from patients undergoing turbinectomy. Specimens were immersed directly in 0.5% protease (Bacillus polymyxa, Sigma Chemical Co., St. Louis, MO) in Earle's balanced salt solution (EBSS) with 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Germany) at 4°C for 16–20 h. Subsequently the epithelial cells could be abraded carefully with scalpels from the lamina propria (0.7–1 × 10^5 cells/cm^2) and isolated by pipetting with 10 mL culture medium. The resulting suspension was centrifuged at room temperature (300 rpm) for 7 min and resuspended in 10^3 cells/mL culture medium, which consisted of Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 10% fetal calf serum (FCS, Biozol, Germany), 1% non-essential amino acids (Gibco, Germany), 1% L-glutamine (Gibco, Germany) and 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were seeded at densities of 10^5–10^6 cells/cm^2 on plastic 6-wells (Nunc, Germany) for characterisation and metabolism studies and cultivated at 37°C in an atmosphere of 10% CO_2 and 95% relative huminity. For transport studies the cells were seeded at equal densities on polyethyleneterephtalat filters (3.8 cm^2), kindly provided by Becton-Dickinson, which were fixed in self-made filter holders and placed in Nunc 6-wells. The medium was changed every third day.

Attachment and proliferation of the cells were observed through an inverse phase contrast microscope (Nikon) with a ×100 magnification.

Actin Staining

F-actin was stained using FITC-labeled phalloidin

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0724-8741/95/0400-0565$07.50/0 © 1995 Plenum Publishing Corporation

(Sigma, Germany) previously described by Anderberg *et al.* (20). Briefly, 10 μL of the stock solution (200 U/mL phalloidin in methanol) was evaporated and redissolved in 400 μL of 0.2 M phosphate-buffered saline, pH 7.4 (PBS). The monolayer, grown on glass slides was rinsed three times with PBS, fixed for 10 min in 4% formaldehyde in PBS, rinsed again and treated with 1% Triton X-100 (Gibco) on ice for 5 min. After repeated washings and air-drying, the monolayer was stained with FITC-phalloidin under light exclusion for 20 min. The sample was rinsed twice with PBS and examined under a Zeiss fluorescent microscope (Germany) with a ×500 magnification.

Microscopic photographs were taken with a Contac RT-SII camera (Germany) and Fuji 100 ASA films.

Scanning Electron Microscopy

Monolayers were washed twice with PBS and fixed in 2.5% glutardialdehyde in 0.15 M cacodylate buffer, pH 7.4 at room temperature for 2 h. The samples were postfixed in 1% osmium tetroxide in the same buffer at room temperature for 1 h. Dehydration was achieved by a graded series of ethanol and critical point drying. Subsequently the samples were sputter-coated with platinum and observed and photographed in a Tesla scanning electron microscope, Type BS 300 (CSSR).

Metabolic Studies

Confluent monolayers, cultivated in Nunc 6-well plates were used at day 11 after seeding. They were rinsed with PBS and allowed to equilibrate in Hank's balanced salt solution (HBSS) containing 15 mM glucose, pH 6.7 for 15 min. Leu-Enkephalin (115 ng/mL) or Met-Enkephalin (1.3 mg/mL) both from Bachem (Germany) were dissolved in HBSS and 1.0 mL was added to the apical side of the monolayer. 100 μL samples were withdrawn at 30, 60, 90 and 120 min and immediately diluted with 500 μL of 0.1 M citric acid to quench further hydrolysis. Analysis was carried out by HPLC.

HPLC

Enkephalins and their metabolites were separated on a reverse-phase column (Lichrospher 100, RP 18, 250x4 mm, Merck) using a mobile phase of 23% acetonitril and 77% of 0.01 M phosphate buffer, pH 2.0. The HPLC instrumentation used was from Hitachi, Merck (Germany) and data acquisition and integration were performed by Millennium 2010 software (Millipore, Waters, Germany). With a flow of 1.1 mL/min and a detection wavelength of 220 nm retention times were as follows: Leu-Enkephalin: 13.0 min; Des-Tyr-Leu-Enkephalin: 8.6 min; Met-Enkephalin: 7.5 min and Des-Tyr-Met-Enkephalin: 5.3 min.

Data Treatment

The disappearance rates of enkephalins and the appearance rates of the metabolites were calculated from the slope of the regression line describing the amount of enkephalin resistant respectively metabolized versus time, which was linear up to 120 min, following pseudo zero-order kinetics.

Results are expressed as the mean of three determinations ± standard deviations and statistical analysis was performed using two-sided independent *t*-test.

Transport Studies

For transport studies confluent cell monolayers, grown for 19 days on polyethyleneterephtalate filters (0.4 μm pore size, Becton-Dickinson) were used. The transport model consists of two glass chambers separated through the filter covered with cells. The solution in the chambers could be mixed by magnetic stirring bars and was maintained at 37°C in a standard side-by-side diffusion chamber.

Two model substances of different molecular weights were dissolved in PBS, pH 7.0 containing 10 mM glucose (transport buffer): FITC-labeled dextran (MW 4400, FD4, Sigma) in a concentration of 200 μg/mL and sulforhodamine (MW 607, SR 101, Sigma) in a concentration of 20 μg/mL.

Monolayers rinsed with transport buffer were allowed to equilibrate in the same buffer for 15 min before they were placed in the diffusion chamber, 4.0 mL of the drug solution, containing both substances was added to the apical side of the monolayer, 0.5 mL samples were withdrawn at different time intervalls from the basolateral chamber and replaced by fresh buffer. The amount of model compounds transported was determined by a Perkin Elmer fluorescence spectrophotometer (Model SF-100) at emission/excitation wavelengths of 490/515 nm for FD 4 and 586/610 nm for SR 101. Standard curves run in the presence and absence of each fluorochrome showed no interference for measuring both fluorochromes in the same solution.

Data treatment

Effective permeability coefficients were calculated from the receiver compartment concentrations and the following relationship:

$$P_{\text{eff}} = \frac{V_R}{A\ C_0}\frac{dc}{dt}$$

where V_R is the volume of the receiver compartment, A is the membrane surface area (1.13 cm^2), C_0 is the initial donor concentration of solute and dc/dt is the slope of the regression line describing the cumulative receiver concentration versus time.

Results are expressed as the mean of 5 experiments ± standard deviations and statistical analysis was performed using two-sided independent *t*-test.

RESULTS AND DISCUSSION

Cell Isolation, Attachment and Growth

Nasal tissue specimens are difficult to obtain routinely for research purposes, since most of the biopsies are used for diagnostic examinations and yield only very limited amounts of material. We tried to isolate epithelial cells from pharyngeal tonsils which are covered by the same columnar epithelium as the nasal cavity. These tissue samples are easily obtained from tonsillectomy. Unfortunately tonsils are usually degenerated by chronic inflammation and therefore ciliated cells are scarce. Furthermore, we were unable to con-

trol the bacterial overgrowth of the monolayers in culture and therefore this approach was abandoned. Tissue samples of turbinates from adults undergoing corrective surgery alleviating nasal obstruction were found to be suitable for cell-culture purposes. By placing the tissue directly after excision into 0.5% (g/v) protease solution, bacterial contamination was significantly reduced. After gently separating human nasal epithelial cells from the basal membrane by scraping with a scalpel and isolation by pipetting, the cell viability was >85% as determined by the trypan blue exclusion test.

The cells were seeded at densities of 10^5–10^6 cells/cm^2 and attached within 24 h on untreated Nunc 6-wells. Usually collagen coated support materials are thought to increase cell attachment and proliferation, since collagen is a part of the lamina propria. Hamster tracheal epithelial cells grown on collagen films proliferate poorly and became squamous as noted by Lee *et al.* (11). They obtained a better differentiation of these cells by using collagen gel as attachment surface due to its three-dimensional structure. An improvement of cell growth and differentation by cultivating human nasal epithelial cells on this gel was not observed in comparison to untreated supports (15). This is confirmed by our previous studies where we did not find any differences in cell growth and proliferation between untreated supports, collagen films or collagen gels (21).

Accordingly, we used a standard medium without any growth supplements: DMEM with 10% FCS. Unlike Wu *et al.* (15) we did not observe an inhibition of airway epithelial cell growth by FCS. On the other hand, growth stimulating factors, such as transferrin, insulin, epithelial growth factor (EGF) or cholera toxin were used by many investigators (11,12,14–16). These factors did not to improve human nasal epithelial cell growth and differentiation in our hands. Similar or even better results were obtained with the relatively simple cell culture medium composition (21).

Visible cell colonies were formed after 1 day and these colonies grew into an optical tight monolayer after 6–8 days under our cultivation conditions and remained unchanged until 20–22 days after seeding before the cells began to detach.

Phase contrast microscopy at that time point revealed the cuboidal surface of the cells, indicative for the epithelial origin of the cultured cells. In fact, fibroblasts were not observed in our cultures, although we did not use preventing methods like preplating on petri dishes (17) or cultivation at 34°C (18).

Morphology and Characterisation

Dome formation, caused by secretion of solutes and water through the basolateral membrane, appeared 8 days after seeding, suggesting ion transport activities and the development of tight junctions between neighboring cells of the monolayers, as shown in Figure 1.

During the entire cultivation time of 21 days actively beating cilia could be observed on the apical side of the cells. These ciliated cells occurred frequently in clusters within the monolayer or they were located on the top of the domes. Recently Jorissen (17) determined the regeneration of cilia on human nasal epithelial cells cultured in suspension, where they formed cellular aggregates which are comparable to domes.

Actin Staining

The presence of tight junctions was also demonstrated using FITC-labeled phalloidin. Phalloidin attaches to actin within the cells which allows visualization by fluorescence microscopy. All cultured nasal cells showed intensive staining of actin fibers close to the cellular junction as shown in Figure 2A. Furthermore a tight network on the apical side of the cells was observed, the so called terminal web, which is

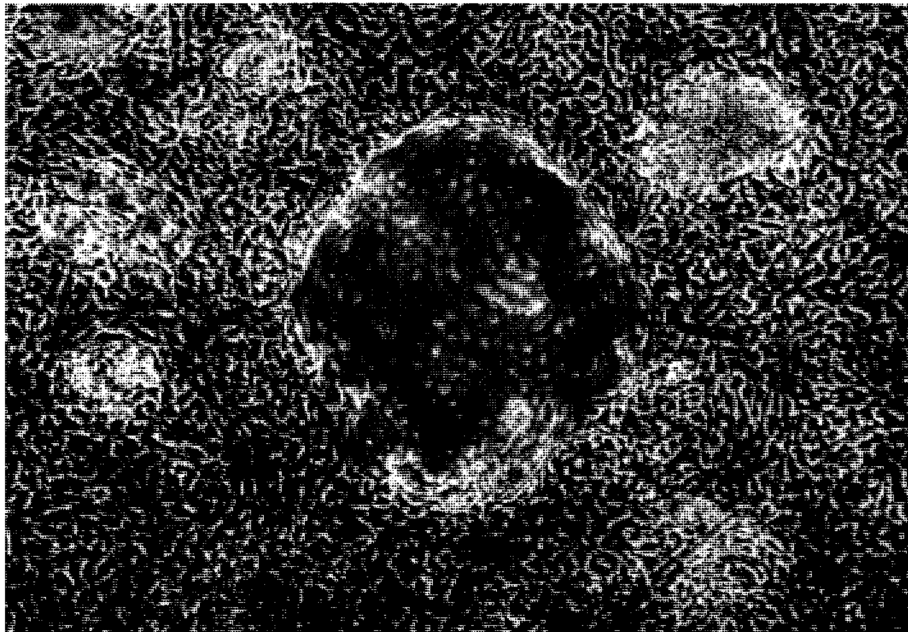

Fig. 1. Dome formation of human nasal epithelial cells after 8 days in culture, indicating the development of tight junctions (100×).

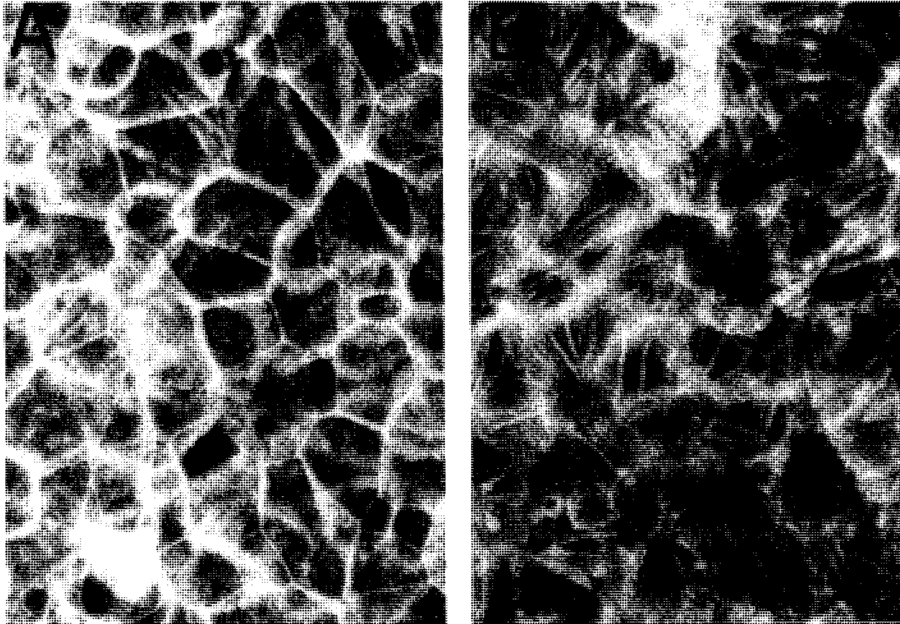

Fig. 2. Actin staining of the monolayers after 8 days (500×). A: Tight junctions, appearing as a "fluorescent belt." B: Terminal web.

another indicator for well differentiated epithelial cells (Fig. 2B).

Scanning Electron Microscopy (SEM)

SEM studies of nasal cells after 6 days in culture generally revealed three different types of epithelial cells. Most prominent (~90% of the monolayer) were cells with microvilli at the surface, forming a coherent monolayer. Ciliated cells could be observed as well, covering approximately 5% of the surface of the monolayer (Fig. 3). This is only 1/3 of cilia normally present on the turbinate epithelium in humans (22). Nevertheless, compared to other human nasal epithelial cell cultures, where ciliated cells disappeared after 7 days in culture (15,17), we noted beating cilia up to least 21 days. Potential ciliotoxic effects of drugs or absorption enhancers, like bile salts could be determined in our in-vitro culture model parallel to transport studies.

The third cell type, rarely observed, were secretory cells identified by cell surface protrusions (data not shown),

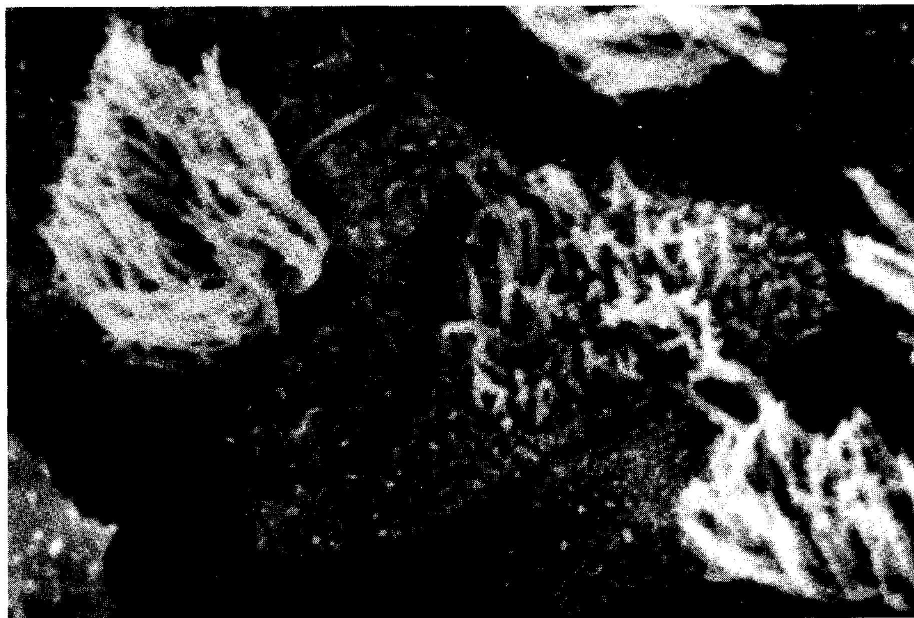

Fig. 3. Scanning electron micrograph of a human nasal epithelial cell monolayer after 6 days in culture showing ciliated cells (4500×).

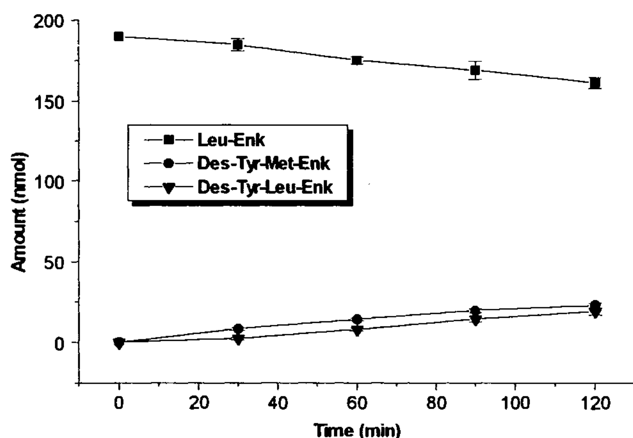

Fig. 4. Disappearance rate of Leu-Enkephalin and appearance rates of Des-Tyr-Leu-Enkephalin and Des-Tyr-Met-Enkephalin respectively upon incubation in nasal epithelial monolayers of 1 cm^2 incubation area. Symbols represent the mean of three determinations ± standard deviation.

which suggested the presence of intracellular granules (11). The composition of the mucus was not determined. Mucin producing goblet cells have not been detected in human nasal cell cultures yet, suggesting that these cells are difficult to cultivate under in-vitro conditions. The main purpose of our study was to investigate transport and metabolism through monolayers and therefore, no attempts were made to increase proliferation of goblet cells.

Metabolic Studies

Aminopeptidases cleaving the N-terminus of oligopepides are frequently found in the nasal mucosa (23). To determine whether nasal epithelial cells retain their enzymatic activity when grown on plastic supports, we studied the metabolism of two well known enkephalins: Leu-Enkephalin (Tyr-Gly-Gly-Phe-Leu) and its analogue Met-Enkephalin (Tyr-Gly-Gly-Phe-Met). As expected, these penta-peptides were cleaved to Des-Tyr-Leu-Enkephalin or Des-Tyr-Met-Enkephalin respectively. The time course of Leu-Enkephalin metabolism is shown in Figure 4, suggesting a pseudo zero-order kinetic behaviour, probably due to substrate saturation. Both enkephalins were not hydrolized in buffer over 120 min.

Zero-order rate constants for the disappearance of Leu-Enkephalin was 0.243 ± 0.028 nmol/min and for the appearance of Des-Tyr-Leu-Enkephalin 0.167 ± 0.023 nmol/min per 1 cm^2 cell surface area. From the ratio of these rate constants we determined, that only 69% of the metabolized Leu-Enkephalin reappeared as Des-Tyr-Leu-Enkephalin. The missing 31% of Des-Tyr-Leu-Enkephalin were probably further degraded. Hussain *et al.* (9) examined the metabolism of Leu-Enkephalin in the perfusate of the nasal cavity in rats. Recalculating their results, by assuming a surface area of 11 cm^2 in the rat nasal cavity, we obtain a reasonable agreement of our data for the formation of Des-Tyr-Leu-Enkephalin, as shown in Table I.

Des-Tyr-Met-Enkephalin yielded a slightly higher but not significantly different ($p = 0.05$) appearance rate constant of 0.19 + 0.016 nmol/min. These differences in metabolic degradation of enkephalins were previously observed in Caco-2 cells in our laboratory (24).

Transport Studies

The nasal mucosa is known to form a tight absorption barrier especially for peptides with a molecular weight above 1000 Da (25). Although we observed well developed tight junctions, a quantification of the barrier function of the cultivated human nasal monolayer is necessary. For this reason, we studied transport rates of FD 4 (MW 4400) and SR 101 (MW 607) through nasal epithelial monolayers grown on membranes in comparison to blank membranes, as shown in Figure 5.

Both substances showed a highly significant ($p < 0.0001$) decrease in transport rates due to the cell monolayer. The permeability coefficients, presented in Table II, are comparable to rat in-vivo P_{eff} values when the calculated absorption constants were converted to a hypothetical monolayer (26). The rate-limiting step is the transport through the cell monolayer. In accordance with McMartin *et al.* (25) we obtain a significantly higher ($p < 0.05$) effective permeability coefficient for SR 101, compared to FD 4 as would be expected considering their molecular masses.

We conclude, that SR 101 is a model compound poorly

Table I. Amounts of Enkephalin-Metabolites Appearing After Different Time Intervals in the Perfusate, Respectively, in the Apical Solution[a]

	In-Situ Rat Nasal Cavity[b] Des-Tyr-Leu-Enk (nmol)	In-Vitro Nasal Epithelial Cells: Des-Tyr-Met-Enk (nmol)	In-Vitro Nasal Epithelial Cells: Des-Tyr-Leu-Enk (nmol)
30 min	~15	8.3 ± 0.8	2.4 ± 0.7
60 min	~23	14.2 ± 0.6	8.0 ± 0.8
90 min	~24	22.9 ± 1.3	14.3 ± 1.7
Zero-order rate constants (nmol/min)	–	0.19 ± 0.02[c]	0.17 ± 0.02

[a] Comparison between in-vivo and in-vitro values per cm^2 incubation area expressed as mean of 3 determinations ± standard deviation.
[b] Values are calculated from data given by Hussain (9).
[c] Not significantly different at $p = 0.05$ related to Des-Tyr-Leu-Enk results using two-sided independent *t*-test.

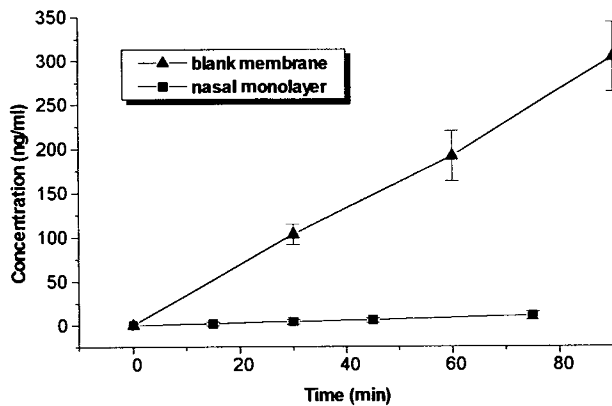

Fig. 5. Time course of SR 101 transport through nasal monolayers and blank filters. The apical concentration was 20 μg/mL. Data expressed the mean of five determinations ± standard deviation.

permeating the nasal mucosa and is therefore a suitable permeability marker for monolayer integrity. Schasteen et al. used SR 101 previously for this purpose (27). Smaller peptides which are known to be well absorbed from the nasal epithelium under in-vivo conditions, such as thyrotropin-releasing hormone (28) are currently under investigation.

Conclusion

In summary, we developed an in vitro model for the human upper airway tract consisting of human nasal epithelial cells in primary culture. When seeded in high densities these cells grew rapidly to a confluent monolayer, expressing tight junctions and beating cilia for at least 21 days. Only few goblet cells were observed. Our data suggest, that this primary culture model is very similar in structure to natural human nasal epithelium. In contrast to previous reports (15–18) we succeeded to culture human nasal epithelial cells using relatively simple media. Aminopeptidase activities of our cultured nasal epithelial cells were comparable to in-situ data. In addition, transport studies demonstrated the formation of tight barrier in the monolayer. Further studies with this relatively simple cell culture model to characterize nasal transport and metabolism of peptides appear to be very promising.

Table II. Effective Permeability Coefficients for FD 4 and SR Through Human Nasal Monolayers and Blank Membranes, Compared with in Vivo Rat Data

	P_{eff} (cm/sec × 10^{-7})[a]		
	FD 4	SR 101	Phenol red
Blank membranes	30 ± 8	82 ± 6	–
Human nasal monolayers	1.8 ± 0.4***	4.2 ± 1.8***	–
In-vivo rat data[b]	~0.13	–	~1.2

[a] Values are means ± standard deviations for 5 determinations and two-sided independent *t*-test results are expressed as differences related to blank membranes (***$p < 0.0001$).

[b] Values are calculated from in-vivo experiments conducted by Yamamoto (26).

ACKNOWLEDGMENTS

We would like to thank Drs. W. Schweckendiek, E. Nolte, P. Nölling, W. Schultheis and the nursing staff of the ORL hospital Schweckendiek, Marburg, for their kind collaboration in obtaining the nasal turbinate tissue. Furthermore we are indepted to Dr. Böhm, Charité, for personal communications and Ms. Weise, Charité, for her kind assistance in the SEM work.

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