Serially Passaged Human Nasal Epithelial Cell Monolayer for *in Vitro* Drug Transport Studies

Jin-Wook Yoo,[1] You-Sun Kim,[2] Sun-Hee Lee,[1] Min-Ki Lee,[2] Hwan-Jung Roh,[2] Byung-Hak Jhun,[1] Chi-Ho Lee,[1] and Dae-Duk Kim[1,3]

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Purpose. To evaluate the feasibility of using a serially passaged culture of human nasal epithelial cell monolayers on a permeable support for *in vitro* drug transport studies. The optimum conditions for passaged culture as well as the correlation between the transepithelial electrical resistance (TEER) value and drug permeability (P_{app}) were evaluated.

Methods. Fresh human nasal epithelial cells were collected from normal inferior turbinates and were subcultured repeatedly in serum-free bronchial epithelial cell growth media (BEGM) in petri dishes. The subcultured cells of each passage were seeded onto permeable supports at 5×10^5 cells/cm^2 and grown in Dulbecco's modified Eagle medium (DMEM). Morphologic characteristics were observed by scanning electron microscopy (SEM). To verify the formation of tight junctions, actin staining and transmission electron microscopy (TEM) studies were conducted. In the drug transport study, [^{14}C]mannitol and budesonide were selected as the paracellular and the transcellular route markers, respectively.

Results. Serially passaged cells were successfully cultured on a permeable support and showed significantly high TEER values up to passage 4. After 14 days of seeding, SEM showed microvilli, and protrusions of cilia and mucin granules were detected by TEM. The paracellular marker [^{14}C]mannitol showed a nearly constant permeability coefficient (P_{app}) when the TEER value exceeded 500 $\Omega \cdot cm^2$ regardless of the passage number. However, as expected, budesonide showed a higher permeability coefficient compared to [^{14}C]mannitol and was less affected by the TEER value.

Conclusions. Human nasal epithelial cell monolayers were successfully subcultured on a permeable support up to passage 4. These cell culture methods may be useful in high-throughput screening of *in vitro* nasal transport studies of various drugs.

KEY WORDS: human nasal epithelial cell culture; serially passaged culture; tight junction; *in vitro* drug transport; transepithelial electrical resistance; mannitol; budesonide.

INTRODUCTION

Drug administration via nasal route has interested many researchers because it is an attractive alternative to oral administration and injection. Its merits include painless application, avoidance of first-pass elimination, and potentially high systemic bioavailability. Drug administration for local effects is also possible for nasal diseases such as rhinitis. *In vivo* animal models (1) and *in vitro* models using excised tissue (2–5) have been proposed for use in nasal drug transport studies. However, because of the difficulty of obtaining intact excised tissue (6), *in vitro* cell culture models (7–14) are being developed. Cultured nasal cells can be applied in drug transport and metabolism studies because they can express important biologic features (e.g., tight junctions) that resemble those of an *in vivo* system. Moreover, easy control of experimental conditions as well as separation of the permeation step from the subsequent absorption cascade (10) is possible. A relatively simple primary culture condition using human nasal epithelial cells for *in vitro* drug transport studies has been established by Werner *et al.* (7,8). This culture method has been applied in transport and metabolism studies of insulin and peptides (11,13,14).

Despite its advantages, however, it is difficult to obtain adequate amounts of human nasal cells because most of the biopsies are used for diagnostic examinations and yield only very limited amount of tissue. The limited resources for *in vitro* primary cell culture has been a serious obstacle for conducting diverse experiments, and thus, high-throughput screening studies have not been possible. Other limitations of primary cell culture methods include contamination with pathogens and large donor-to-donor variability (15). To overcome these limitations, a serially passaged system can be considered as an alternative. Previously, studies on the development of a passaged culture of human respiratory epithelial primary cells had been conducted to investigate physiologic characteristics, such as mucin secretion (15-19). However, there are no reports where serially passaged cells have been cultured on a permeable support for *in vitro* drug transport studies. The formation of tight junctions would be the most essential factor to be considered for transport studies of drugs using the serially passaged nasal epithelial cell monolayer. Herein we report on results of serially passaged human nasal cells with the purpose of conducting *in vitro* drug transport studies. Morphologic characterization of the monolayer on a permeable support using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and assessment of monolayer permeability using transepithelial electrical resistance (TEER) were used. In addition, the utility of these cell cultures was assessed for their use in *in vitro* drug transport studies by evaluating their permeability to [^{14}C]mannitol and budesonide, hydrophilic and lipophilic model permeants, respectively.

MATERIALS AND METHODS

Cell Culture: Isolation, Expansion, and Subculture

Nasal specimens were obtained during surgery from inferior turbinate mucosa of patients suffering from septal deviation or chronic sinusitis. The tissues were treated with 1.0% Pronase (type XIV protease, Sigma, St. Louis, MO) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient F12 (DMEM/F12) supplemented with penicillin (50 IU/mL) and streptomycin (500 μg/mL) for 16 to 20 h at 4°C. Dissociated epithelial cells were washed three times with DMEM/F12 containing antibiotics and then suspended in the same medium supplemented with antibiotics and 10% fetal bovine serum. Cells were preplated on a plastic dish at 37°C for 1 h in order to eliminate fibroblasts, endothelial cells,

[1] College of Pharmacy, Pusan National University, Pusan 609-735, South Korea.
[2] College of Medicine, Pusan National University, Pusan 602-739, South Korea.
[3] Current address: College of Pharmacy, Seoul National University, Seoul 151-742, South Korea.
[4] To whom correspondence should be addressed. (email: ddkim@snu.ac.kr)

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and myoblasts by differential attachment to the bottom of the plastic well. Suspended epithelial cells were frozen using DMSO (dimethyl sulfoxide) and stored in a liquid nitrogen tank for future uses. Frozen passage-1 stocks were thawed and then were seeded at 3×10^4 cells/dish (500 cells/cm^2) in plastic tissue culture dishes. The culture medium was the serum-free bronchial epithelial growth medium (BEGM, Clonetics Corp.), containing hydrocortisone (0.5 μg/mL), insulin (5 μg/mL), transferrin (10 μg/mL), epinephrine (0.5 μg/mL), triiodothyronine (6.5 μg/mL), gentamycin (50 μg/mL), amphotericin B (50 μg/mL), retinoic acid (0.1 ng/mL), and epidermal growth factor (0.5 ng/mL, human recombinant) (all supplied by Clonetics Corp. San Diego, CA), and further supplemented with epidermal growth factor (1.5 ng/mL, Sigma), bovine serum albumin (1.5 μg/mL, Sigma), and bovine pituitary extract (1% vol/vol, Pel-Freez Biologicals, Rogers, AR). Culture incubator was maintained at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The medium was changed every 2 days.

When cultures reached approximately 70–80% confluency, the cells were detached with 0.1% trypsin-EDTA (Gibco BRL, Gaithersburg, MD) and were seeded at a density of 5×10^5 cells/cm^2 onto permeable supports (Costar 12-mm Transwells®, polyester 0.4 μm, Cambridge, MA). Culture medium was DMEM (Gibco BRL, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 ng/mL EGF, 1% nonessential amino acids, and 1% L-glutamine) in both the apical and basolateral sides at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity, which was changed after 24 h of seeding and then every 2 days. Remaining cells after seeding were again subcultured on a plastic culture dish at a density of 3×10^4 cells/dish for next passage in BEGM, as mentioned above.

Actin Staining

F-actin was stained with rhodamine-labeled phalloidin (20). The passage-2 and -4 human nasal cell monolayers grown on permeable supports for 5 days were rinsed three times with PBS and were fixed for 10 min in 3.7% formaldehyde in PBS on ice. After rinsing three times with PBS, they were treated with 1% Triton X-100 (Gibco BRL) on ice for 5 min. Then, they were rinsed with PBS twice and were air-dried. The cells were then stained with rhodamine-labeled phalloidin (2 U/400 μL PBS) for 20 min in the dark. The monolayers were rinsed again with PBS three times, and the filters were then mounted on glass slides with gelvatol and covered with a coverslip. Then, they were examined under a fluorescent microscope (Axioplan2, Zeiss, Germany) at a ×200 magnification.

Electron Microscopy

TEM and SEM were performed to characterize the morphology of passage-2 and -4 nasal epithelial cells grown on permeable supports for 5 days. For SEM, cells were rinsed with 0.1 M PBS and fixed in 2.5% glutaldehyde for 2 h at 4°C. Specimens were rinsed with PBS and treated with 1% osmium tetroxide for 1 h at 4°C. Subsequently, the specimens were dehydrated through serial ethanol solutions (50, 60, 70, 80, 95, and 100%) and finally in hexamethyldisilazane. The specimens cut from the permeable support were coated with gold in a sputter coater (E-1030, Hitachi, Pleasanton, CA) and then were examined by the scanning electron microscope (S-4300, Hitachi).

For TEM, the specimens were fixed as for SEM. After dehydration, the specimens were embedded in Epon 812. Semithin sections (80 nm in thickness) were stained with toluidine blue and observed by light microscopy. Appropriate areas were selected, and ultrathin sections were made. They were treated with uranyl acetate at room temperature for 6 min and treated with lead citrate for 3 min. These samples were examined in a JEM 1200 EX II electron microscope (Jeol, Japan).

Transepithelial Electrical Resistance Measurement

The integrity of the monolayer of nasal cells was determined by measuring TEER values using a voltohmmeter (EVOM, WPI, Sarasota, FL) equipped with Endohm electrodes. The TEER of the monolayer was calculated by subtracting the TEER value of the permeable support from total TEER value. The data were calculated as means ± SD of at least three observations.

Transport Experiments

Transport medium (Hank's balanced salt solution supplement with 15 mM glucose and HEPES buffer, pH 7.5) was used for transport studies of [^{14}C]mannitol and budesonide across the nasal epithelial cell monolayers. The cell monolayers were first rinsed twice with transport medium and were preincubated with the same medium for 15 min at 37°C. After measuring the TEER value, each transport experiment was initiated by adding 0.4 mL of transport medium containing budesonide (10 or 20 μg/mL, Sigma) or 4 μM [^{14}C]mannitol (specific activity of 56.0 Ci/mmol, Amersham, Arlington, IL) on the apical side, and 1.0 mL of blank transport medium in basolateral side. At predetermined time intervals (10, 20, 30, 45, and 60 min), 1.0-mL samples were withdrawn from the basolateral side and quickly replaced with equal volumes of fresh transport medium. At the end of experiments, apical solutions were also taken to determine the mass balance. After budesonide transport experiments, the TEER value was determined to check the integrity of the cell monolayers.

For analysis of radioactive [^{14}C]mannitol samples, 0.3 mL of each sample was mixed with 2.0 mL scintillation cocktail (Ultima Gold, Packard, The Netherlands) and was analyzed by the Tri-Carb 2200CA liquid scintillation counter (Packard Instrument, Meriden, CT). The concentration of budesonide in samples was determined using a HPLC system equipped with a binary pump system (Gilson Model 305 and 306) and an automatic injector (Gilson Model 234). Merck C_{18} LiChroCART 125-4 column (5 μm, 125 × 4 mm, Merck, Darmstadt, Germany) was used as an analytic column at ambient temperature. A mobile phase of 70% methanol and 30% water was used. With a flow of 1.0 mL/min and a detection wavelength of 242 nm, retention time was about 4.2 min.

The apparent permeability coefficient (P_{app}, cm/s) of mannitol and budesonide was estimated using the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{A \cdot C_0}$$

where dQ/dt is the solute flux obtained from linear regres-

sion, A is the surface area of a permeable support (1.13 cm^2), and C_0 is the initial drug concentration.

RESULTS

Characterization of Passaged Human Nasal Epithelial Cell Monolayer

Actin Staining and Transmission Electron Microscopy

Tight junctions are characterized as the fusion of contiguous cell membranes located in the apical side. There are many molecular components of the tight junction, such as occludin, acludins, JAM (junctional adhesion molecule), zonula occludens, and actin (21). Among these components, the perijunctional actin is known to play a major role in controlling paracellular permeability (21,22). The stained actin contours, which present a formation of tight junctions, are shown in Fig. 1. Well-formed tight junctions were observed in both passage-2 and -4 cultures. However, the size of each cell was smaller and tighter in passage 2 than those in passage 4.

In TEM studies, tight junctions were clearly observed in both passage-2 and -4 monolayers grown for 5 days, as shown in Fig. 2 a,c (arrow A). Especially, in passage 2, desmosomes were also clearly observed (Fig. 2a, arrow B). Mucin granules were observed only after 14 days of seeding (Fig. 2b,d, arrow C).

Scanning Electron Microscopy

SEM studies were conducted 5 days after seeding in a similar manner to the TEM studies and are shown in Fig. 3 at ×3000 magnification. In passages 2 and 4, most of the cell surface of the monolayer was covered with microvilli, and cilia were observed in about 5% of the monolayer cells. However, mature and beating cilia were not observed in passage-2 or -4 monolayer after 5 days of seeding.

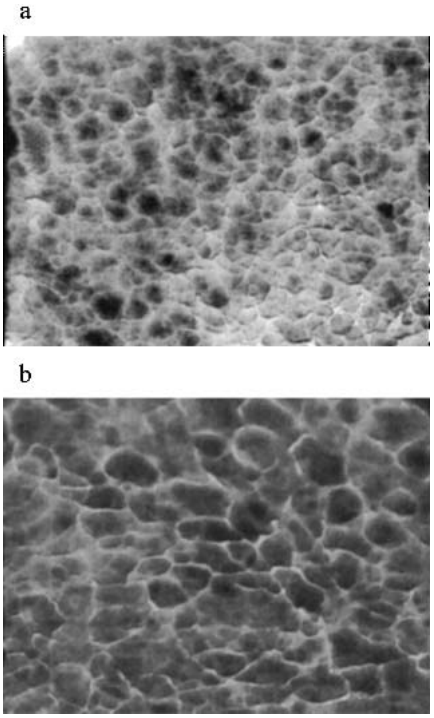

Fig. 1. Actin staining of the passage-cultured human nasal epithelial monolayer, 5 days after seeding on a permeable support. The obvious silhouettes of stained actin filaments are shown (×200 magnification). a, Passage-2 monolayer. b, Passage-4 monolayer.

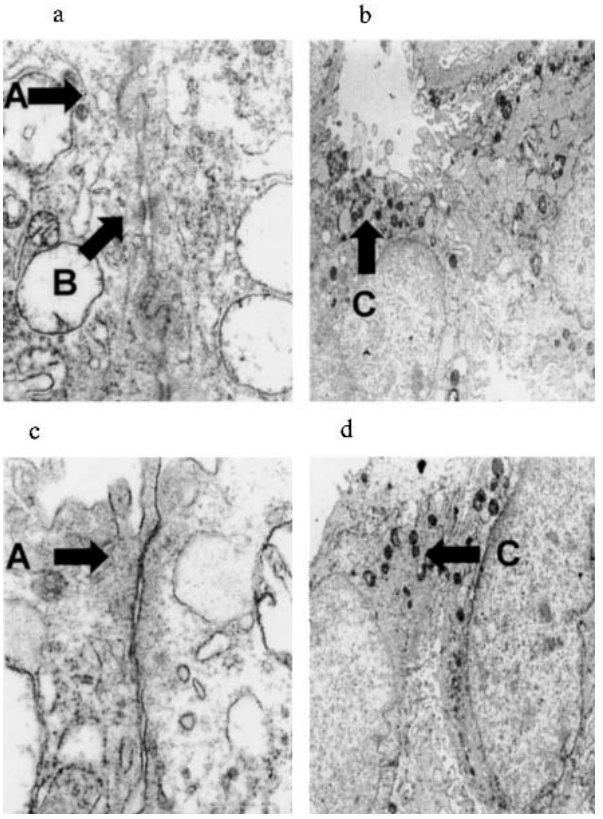

Fig. 2. Transmission electron microscopy (TEM) of the passage-cultured human nasal epithelial monolayer on a permeable support (×40,000 magnification): (a) 5 days after seeding, passage 2; (b) 14 days after seeding, passage 2; (c) 5 days after seeding, passage 4; (d) 14 days after seeding, passage 4. A, tight junction; B, desmosome; C, mucin granule.

Changes of TEER Value

Fig. 4 shows the TEER values of each passage cultured epithelial cell monolayers measured using EVOM for 14 days to determine the effects of the passage number. The maximum TEER values were observed in 2 days after seeding and then rapidly decreased. The maximum values of each passage were 3133 ± 665, 2703 ± 407, and 1235 ± 74 $\Omega \cdot cm^2$ for passages 2, 3, and 4, respectively. In passage-2 cells, TEER value suddenly dropped after the highest value after 2 days but remained over 500 $\Omega \cdot cm^2$ up to 8 days after seeding (Fig. 4a). In passage-3 cells, TEER values were over 500 $\Omega \cdot cm^2$ and lasted for up to 12 days after seeding (Fig. 4b). In passage-4 cells, the maximum TEER value was lower than those of passages 2 and 3. Although the change of TEER appeared to fluctuate (Fig. 4c), TEER values over 500 $\Omega \cdot cm^2$ were observed for 10 days. Passage-5 cells showed the maximum TEER value of 693 ± 80 $\Omega \cdot cm^2$ after 3 days, after which less than 500 $\Omega \cdot cm^2$ was maintained. Cells over passage 6 did not show significant TEER value (data not shown).

TEER vs. Permeability of [^{14}C]Mannitol and Budesonide

In each passage, nasal cell monolayers of various TEER values were selected to conduct transport studies using

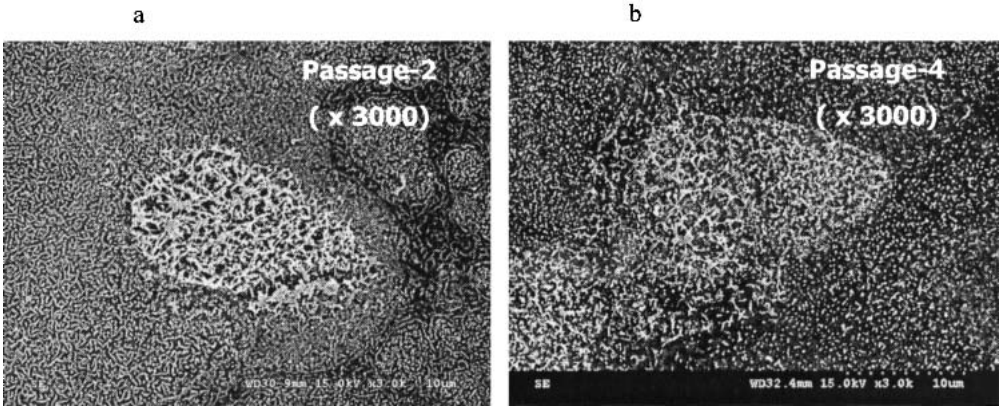

Fig. 3. Scanning electron microscopy (SEM) of the passage-cultured human nasal epithelial monolayer, 5 days after seeding on a permeable support: (a) passage 2 (×3000 magnification); (b) passage 4 (×3000 magnification).

[^{14}C]mannitol, which is known as a paracellular route marker, and then a correlation between TEER and P_{app} of hydrophilic drugs was investigated. It is interesting to note that a nonlinear relationship between TEER values and P_{app} of [^{14}C]mannitol was observed, as shown in Fig. 5. TEER values of around 500 $\Omega \cdot cm^2$ appear to be the critical point corresponding to changes of mannitol permeability. At TEER values less than 500 $\Omega \cdot cm^2$, P_{app} of mannitol rapidly decreased as TEER values increased. Above 500 $\Omega \cdot cm^2$, however, the decrease of P_{app} was remarkably attenuated as TEER values increased, and P_{app} value was almost constant regardless of the TEER value. In addition, it was obvious that no noticeable divergence of P_{app} value existed among passages 2, 3, and 4.

Budesonide (log p = 3.2) was chosen as a model lipophilic drug to determine the trend of the transcellular permeability in various TEER values. As shown in Fig. 5, when 20 μg/ml of budesonide was applied on the apical side of the monolayers with various TEER vaules, the P_{app} of budesonide was always higher than 1.2×10^{-5} cm/s and was not significantly affected by TEER values in passaged nasal cells. Similar to the mannitol transport study, no detectable differ-

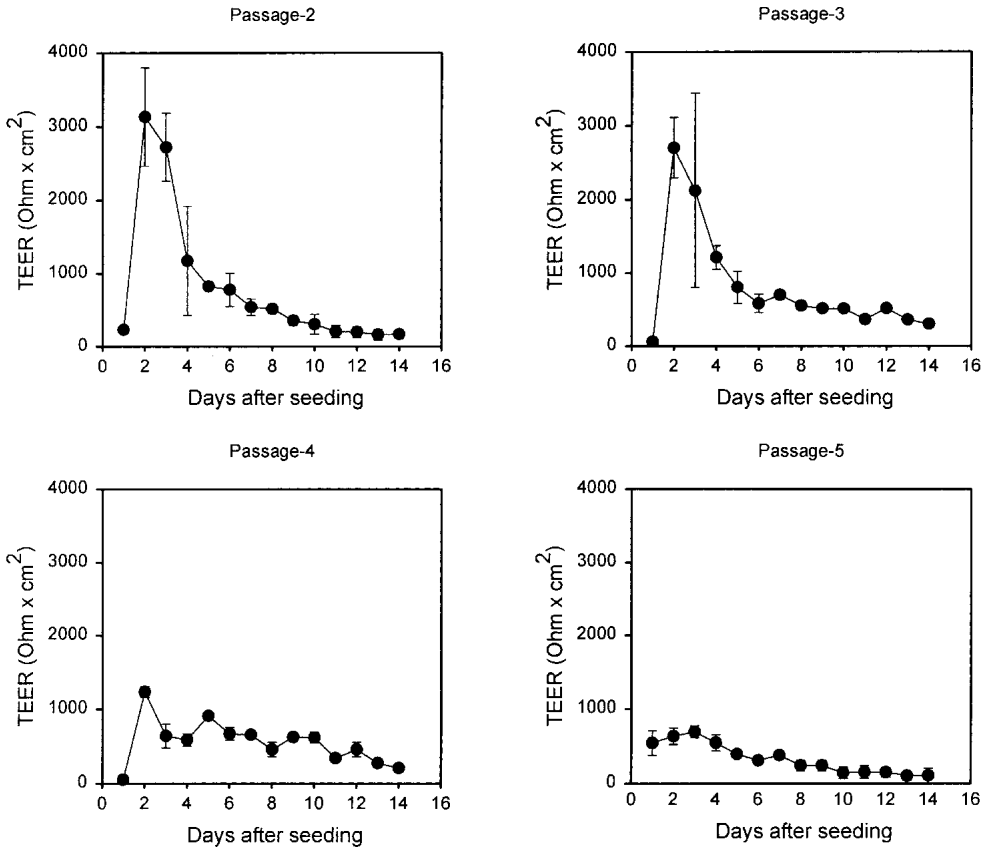

Fig. 4. Changes of transepithelial electrical resistance (TEER) in each passage number over 2 weeks. Each value is the mean ± SD of three to six determinations.

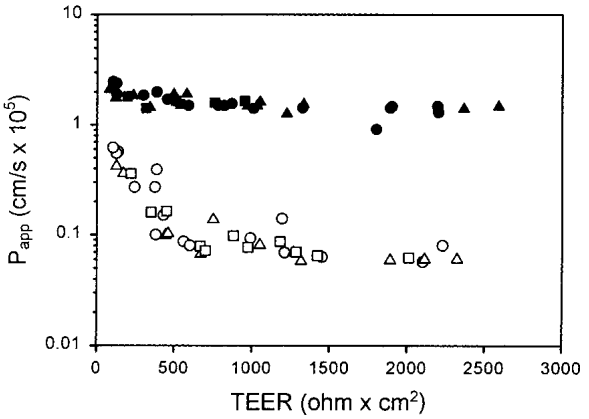

Fig. 5. Relationship between the TEER value of the monolayer and permeability of [^{14}C]mannitol [passage 2 (○), passage 3 (△), passage 4 (□)] and budesonide [passage 2 (●), passage 3 (▲), passage 4 (■)].

ence of P_{app} value was observed among passage numbers. Fig. 6 shows the transport profiles of budesonide when 10 μg/ml or 20 μg/ml was applied on the apical side of the monolayers with higher than 500 Ω·cm^2 TEER value. The P_{app} value was not significantly different between the two concentrations (Table I).

DISCUSSION

Passaged Culture of Human Nasal Epithelial Cell Monolayers

Primary cultured cells have been used for *in vitro* nasal drug transport studies because it was believed that first-passage cells had the most similar characteristics to actual human nasal cells in their morphology, phenotype, and integrity. However, their general application in transport studies has been limited because of the difficulty in handling and obtaining the cells. In this study, the isolated nasal epithelial cells were frozen and stored in a liquid nitrogen tank for future use, as described for tracheobronchial epithelial cells (15). This freezing process makes it possible for any laboratory that has difficulty in accessing clinical facilities with human primary cells to use the cells whenever necessary. Although thawed cells included a few lysed cells, their growth

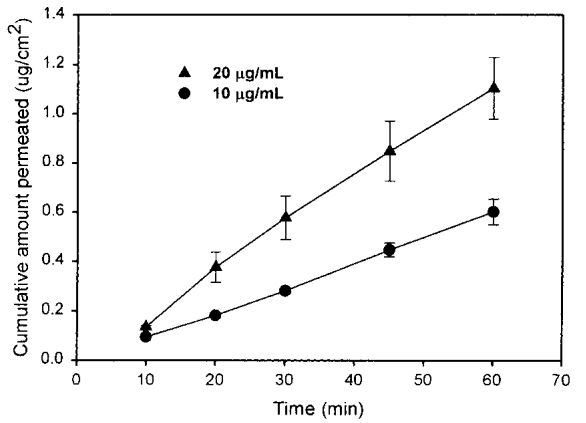

Fig. 6. Transport profiles of budesonide across the passage-2 human nasal cell monolayer with higher than 500 Ω·cm^2 TEER value on day 5.

Table I. Effect of Apical Concentration of Budesonide on the Permeability Coefficient Through the Passage-2 Human Nasal Cell Monolayer on Day 5

Budesonide (μg/mL)	Permeability coefficient (cm/s)	TEER value (Ω · cm^2)
10	1.71 (±0.19) × 10^{-5}	1420 ± 60
20	1.60 (±0.20) × 10^{-5}	1645 ± 120

Each value is the mean (±SD) of triplicate experiments.

and expansion were not different from cultured cells that were not frozen (data not shown).

In order to solve the problem of obtaining enough cells, however, it was necessary to use a serially passaged culture method. Moreover, for a transport study, developing the nasal cells into monolayers on permeable supports was crucial. The most important feature in a monolayer for use in transport studies is the development of tight junctions measured by use of TEER values in this study. Our previous study revealed that selection of the culture media was one the most critical factors for developing tight junctions. Although BEGM is known to be suitable for cell growth (23), the actin staining and [^{14}C]mannitol transport study showed that the formation of tight junctions was not sufficient (data not shown). Therefore, BEGM was selected for the proliferation of the cells on a plastic dish, and DMEM was selected for differentiation of cells on a permeable support, as previously described (7).

Serial culture up to passage 6 was conducted in this study. It took 3 to 5 days for passages 2, 3, and 4 to expand cells confluently in plastic dishes before they were seeded on a permeable support. However, passages 5 and 6 required approximately 10 days, and many of them were also swollen and lysed, which caused the cells to grow improperly on the permeable support. Thus, cells up to passage 4 seemed to be suitable for subculturing.

Characterizations of Passage Cultured Human Nasal Epithelial Cells

The staining of actin filaments is the most commonly used technique to verify the existence of tight junctions. As shown in Fig 1, the outline of stained actin filaments was similar to that reported in the literature (7). The monolayer of passage 2 looked more compact with smaller cells than that of passage 4, which resulted in higher TEER value (Fig. 4). Tight junctions were also clearly observed in the TEM study of passage-2 and -4 cells after 5 days of seeding (Fig. 2a,c). Mucin granules were observed in passage-2 and -4 cells only after 14 days of seeding (Fig. 2b,d). However, because tight junctions seemed to be loosened after 14 days, it was not possible to simultaneously observe both tight junctions and mucin granules (or mucin-secreting cells), which are two characteristics of differentiated nasal cells. In the SEM study, protrusions of cilia and numerable microvilli were observed after 5 days in passage-2 and -4 cells (Fig. 3), similar to what was observed in ALI (air–liquid interface) culture conditions after 7 days of seeding (24).

The effect of passage culture on the changes of TEER value was observed up to passage 5 for 14 days (Fig. 5). TEER values of passages 2, 3, and 4 remained higher than 500 Ω·cm^2

for about 10 days, which implies that it would be suitable to use this passaged culture system for drug transport studies. The maximum TEER values were observed 2 days after seeding and reached approximately 3000 $\Omega \cdot cm^2$ in passage-2 and passage–3 cultures. The short onset time for the high TEER values is an unusual characteristic compared to other culture systems reported in the literature (7–11). In human nasal primary culture using coating membrane (12) and rabbit tracheal primary culture system (25), maximum TEER values were reported to be 1349 ± 508 $\Omega \cdot cm^2$ in 8 days and about 1500 $\Omega \cdot cm^2$ in 7 days, respectively. The prompt formation of tight junction in the serially passaged system, which could be advantageous in drug transport studies, is probably a result of the increase in homogeneity of nasal cells after subsequent subcultures. The lower TEER values in passages 4 and 5 compared to those in passages 2 and 3 may be caused by the decrease of viability causing loss in differentiation ability.

Applications for the *In Vitro* Drug Transport Studies

The feasibility of passage-cultured human nasal epithelial cell monolayers for *in vitro* drug transport studies was confirmed by transport experiments with [^{14}C]mannitol and budesonide at various TEER values. P_{app} values of [^{14}C]mannitol and budesonide at each passage were consistent (Fig. 5), which implies that the model would be applicable to drug transport studies up to passage-4 culture. The permeability coefficient of [^{14}C]mannitol in Fig. 5 was similar to that of porcine nasal mucosa studied at the same TEER value (6). This suggests that a correlation exists between the permeability of serially passaged cells and that of freshly excised intact tissue. Further studies are being conducted in order to investigate the relationship between the *in viv*o and *in vitro* systems.

A nonlinear relationship between P_{app} and TEER values of [^{14}C]mannitol (Fig. 5) was also reported in rat alveolar culture (26), Calu-3 cell line culture (27), and bronchial epithelial cell culture (28), in which the critical TEER value was around 1,200 $\Omega \cdot cm^2$, 300 $\Omega \cdot cm^2$, and 250 $\Omega \cdot cm^2$, respectively. Because hydrophilic drugs are known to permeate through the paracellular route of the nasal epithelial cell monolayer, the complete formation of tight junctions is essential in order to obtain consistent P_{app} value. Based on the [^{14}C]mannitol transport study, TEER values of the monolayer need to be higher than 500 $\Omega \cdot cm^2$ for hydrophilic drugs to obtain a consistent P_{app} value in transport studies.

Contrary to mannitol transport, budesonide showed rather constant P_{app} values regardless of TEER values. The tight junctions of the monolayers do not seem to influence the P_{app} value because the transcellular route is the major transport route of lipophilic drugs (4). When the TEER value was higher than 500 $\Omega \cdot cm^2$, constant P_{app} values were obtained regardless of the budesonide concentration (10 and 20 μg/mL) and passage number (Table I). This suggests that the passage-cultured human nasal cell epithelial monolyers can be used in *in vitro* drug transport studies.

CONCLUSIONS

Passage-cultured human nasal epithelial cell monolayers for *in vitro* drug transport studies were successfully established up to passage 4. Each passaged culture formed a confluent tight monolayer with high TEER values sufficient for use in drug transport studies, although the differentiation of cilia and mucin-secreting cells was not complete. This passaged culture system was used to conduct multiple and reproducible *in vitro* transport studies using limited human nasal cell sources. The passaged culture system established in this laboratory, therefore, could be a solution to overcome the limitations of the existing primary culture system and may be useful for high-throughput screening in drug permeation studies.

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