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In vitro cell culture model for anti-cataract drug penetration studies

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Immortalized human corneal epithelial cells (HCECs) and human lens epithelial cells (HLECs) were cultured *in vitro*. Cells were observed under a phase-contrast microscope and the integrity of cell monolayers was assayed by transepithelial electrical resistance (TEER) determination. The permeability of disulfiram (DSF) through a HCECs monolayer was compared with that of DSF through an excised rabbit cornea. The permeability coefficients of DSF through a HCECs monolayer and excised rabbit cornea were $29.5 \pm 4.8 \times 10^{-6}$ cm/s and $34.7 \pm 5.2 \times 10^{-6}$ cm/s, respectively. Diethyldithiocarbamate (DDC) had high permeability through HLECs monolayer with a permeability coefficient of $44.6 \pm 7.1 \times 10^{-6}$ cm/s. The cytotoxicity of DDC against HLECs was investigated using the trypan blue exclusion test. For a DDC concentration of 5 mmol/l, more than 85% cells were viable. DH3a1 mRNA was expressed in cultured HLECs. The expression of aldehyde dehydrogenase 3a1 (ALDH3a1), which may be be responsible for DSF-DDC conversion, was detected using RT-PCR and agarose gels electrophoresis. These results demonstrate that the permeability of DSF can be detected and intra-ocular drug action may be predicted using the cultured HCEC and HLEC monolayers as model.

1. Introduction

It is very necessary for anti-cataract drugs to penetrate through the lens epithelial monolayer. The cells of the lens epithelial monolayer are essential for growth, differentiation and homeostasis of the whole lens (Harocopos et al. 1998; Kalariya et al. 1998; Hightower 1995). Dynamics of drug in the lens and effect of drug on lens epithelial cells were studied by *in vivo* animal experiments and phacoemulsification of isolated rabbit lens, respectively (Ahmed et al. 1989; Zhang and Li 1995). In our previous report, we prepared disulfiram (DSF) and hydroxylpropyl-β-cyclodextrin inclusion eye drops and evaluated their anti-cataract effects on selenite-treated rats (Wang et al. 2004). At that time, a large number of animals were needed and the experiment was time-consuming. Furthermore it is less reliable to use these results to predict the performance of drug on human eyes due to the essential difference of the eyes on the anatomy and protein expression for humans and animals (Elisa et al. 2001). Compared with animal experiments, a cell culture model could be more effective.

The immortalized human lens epithelial cells (HLECs) line has been reported by Ibaraki et al. 1998. Although some studies on biochemical characteristics of lens epithelial cells have been done with HLECs line, few reports have been found about drug penetration through HLECs monolayer, as well as interaction with the cells and so on. In this study, we performed *in vitro* DSF and DDC transcorneal permeability experiments using cultured cell monolayer models and further investigated the cytotoxity of DDC against HLECs to assure a safety administration of drugs based on the result reported (Li et al. 2005).

2. Investigations, results and discussion

2.1. Cell culture and TEER detection

2.1.1. HCECs culture

Cells were cultured on 6-well transwell inserts precoated with collagen type I and observed under phase-contrast microscope. The morphologic appearance of HCECs culture is shown in Fig. 1. The observations were made for twice individually cultured cells, and the micrographs were similar to Fig. 1.

Transepithedial electrical resistance (TEER) detection results are shown in Fig. 2. It was shown that TEER values rapidly enhanced within 5 days culture time, and then kept almost constant. The TEER results show that the number of HCECs rapidly increased at the beginning and then intercellar junctional complexes began to appear. Therefore, TEER results indicate that the HCECs monolayer could be used after 5 days culture.

2.1.2. HLECs culture

It was reported that cellular behavior modulated by (or associated to) cell-matrix inactions includes growth, differentiation, apoptosis, gene expression and so on (Yan et al. 2002). Lens capsule consists of several kinds of extracellular matrix (ECM) such as collagen type IV, laminin and fibronectin, among which collagen type IV was observed

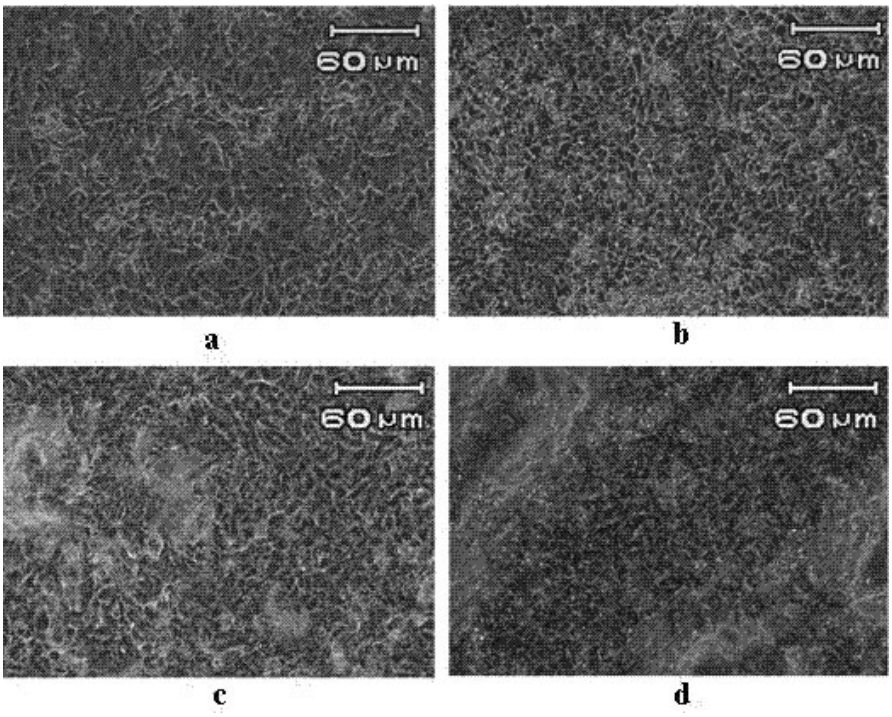


Fig. 1:
Phase-contrast micrographs of HCECs. a) post 4 days culture, b) post 7 days culture, c) post 14 days culture, d) post 21 days culture

at the base of human lens epithelial cells. Collagen type IV is localized almost exclusively at basement membranes which provide the structure support for cells and an anchorage for the neighboring cells and may serve cell attachment, migration and differentiation. Thus, we precoated collagen type IV on the transwell inserts. HLECs were cultured on 6-well transwell inserts precoated with collagen type IV and observed under a phase-contrast microscope. The morphologic appearance of HLECs culture (twice individual culture) was also observed under a phase-contrast microscope and photographed (not shown).

Cells were seeded on 6-well transwell inserts with collagen type IV coating (10 μg/cm^2). Cells were confluent after 4 days culture with the initial seeding density of 2×10^4 cells/cm^2 cultured collagen coated inserts. The cell growth curve is not shown. It takes shape of the typical cells grown curve, reaches the plateau phase after 4 days culture and begins to decline after 7 days culture. Furthermore, during culture, we observed a large number of cells detached from the substrate membrane when the cells were continuously cultured for 4 ~ 5 days. Penetration experiment was performed at the 4th day after seeding, when the amount of the cells reached nearly maximum and a firm reliance both on cell-cell interaction and cell-substrate interaction was formed.

HLECs cells were cultured on polyester transwell inserts with collagen type IV coating. Measured TEER values during cell culture are shown in Fig. 3. Cells monolayer would not be used in this study until mature intercellular junctional complexes appeared. Though cell monolayer cultured on transwell inserts for more than 4 days had greater TEER, the cells were easy to detach from the collagen membrane when rinsing the membrane with penetration medium before the penetration experiment. After 4 days culture, it seems the cells weaken the attachment with collagen membrane, which had a bit more reliance on cell-cell associations rather than cell-matrix associations. Moreover, it has been suggested that when using cultured epithelial cell monolayer for flux studies the optimal TEER value should be one above which the perme-

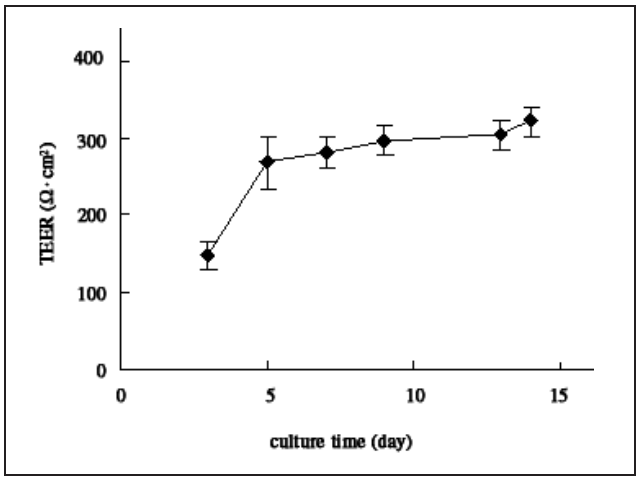


Fig. 2: Effect of HCECs culture time on TEER. Each point represents the mean ± S.D. of 3 wells in transwell inserts

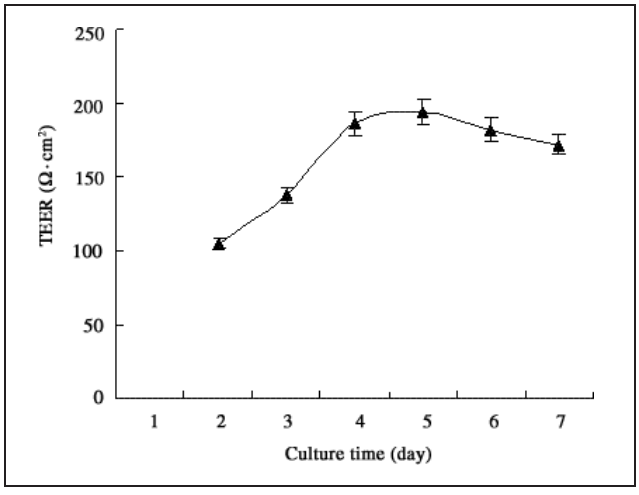


Fig. 3: Effect of HLECs culture time on TEER. Each point represents the mean and S.D. of 3 wells in transwell inserts

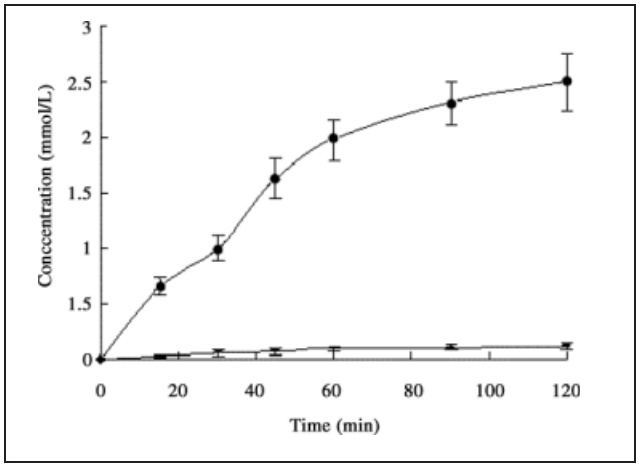


Fig. 4: DSF penetration through HCECs monolayer. • accumulated amount of DDC, – accumulated amount of DSF. Each point represents the mean ± S.D. of 3

ability of the objective compound no longer decreases with increasing TEER (Adson et al. 1994). The amount of drug penetrating through the monolayer increased slowly on the 6th and 7th day compared with 4th day (data not shown), probably because of drug penetration through much more passing route left by the decreased number of cells. Thus, the optimal culture time for performing the penetration experiments was selected as the 4th or 5th day.

2.2. Penetration experiments

2.2.1. DSF penetration through HCECs monolayer

Disulfiram (DSF) is the dipolymer of diethyldithiocarbamate (DDC) and DSF can change into DDC, so DSF and DDC were all detected in the basolateral side during DSF penetrating through HCECs monolayer. The curves of accumulated concentrations of DSF and DDC against time are shown in Fig. 4.

The curve demonstrates that the concentration of DDC is largely higher than that of DSF at each time points. Therefore, we presume that most of the DSF was transformed into DDC while penetrating through HCECs monolayer. Since DSF is the dipolymer of DDC, the total accumulated concentration of DSF in the basolateral side should be equal to the accumulated concentration of DSF plus 50% that of DDC at each time point. In this way, the permeability coefficient of DSF through HCECs monolayer was calculated as $(29.5 \pm 4.8) \times 10^{-6}$ cm/s and shown in the Table 1.

2.2.2. DDC penetration through HLECs monolayer

The accumulated concentration of DDC detected at the basolateral side with time is shown in Fig. 5. We can see the accumulated concentration of DDC increase gradually.

Table: Data analysis of penetration experiment (n = 3)

Samples	(Permeability coefficients ± S.D.) × 10^6 (cm/s)	
	Cell culture model	Isolated rabbit cornea
DSF penetrating through HCECs monolayer	29.5 ± 4.8	34.7 ± 5.2
DDC penetrating through HLECs monolayer	44.6 ± 7.1	–

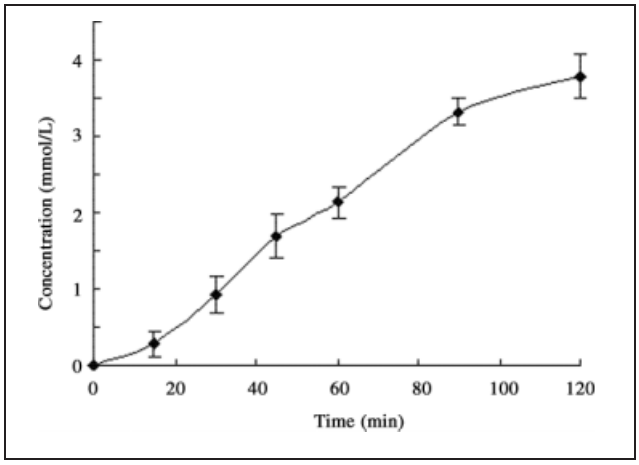


Fig. 5: DDC penetration through HLECs. Each point represents the mean ± S.D. of 3 experiments

The phenomenon is similar to that of drug penetraten through general bio-membrane, which shows that the HLECs monolayer can be used as a model to predict the behavior of drug penetrating into the lens capsule. The permeability coefficient of DDC through HLECs monolayer was calculated as $(44.6 \pm 7.1) \times 10^{-6}$ cm/s is shown in the Table.

2.2.3. Permeability studies with excised cornea

The accumulated amount of DDC detected in the basolateral side with time is shown in Fig. 6.

None but DDC appeared in this permeability studies. Half of the accumulated concentration of DDC represents the total penetrated concentration of DSF at each time point. The permeability coefficient of DSF through excised cornea was calculated as $(34.7 \pm 4.8) \times 10^{-6}$ and listed in the Table. Compared with HCECs monolayer, the permeability coefficient of DSF through excised cornea is a little higher.

2.2.4. Trypan blue exclusion assay

The percent of viable HLECs was calculated from the trypan blue dye exclusion tests and is shown in Fig. 7. Viability of HLECs did not decrease significantly from 12 h to 48 h, which indicates that the cytotoxity of DDC solution with the concentration of 5 mmol/l against HLECs was slight. In addition, for both of HCECs and HLECs, the viability was retained before and after the penetration experiment.

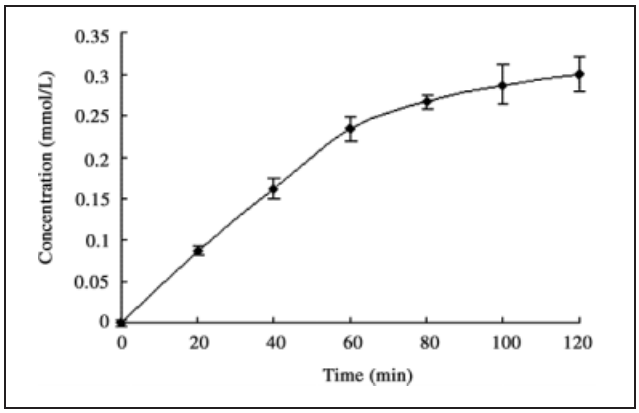


Fig. 6: DSF penetration through excised rabbit cornea. Each point represents the mean ± S.D. of 3 experiments

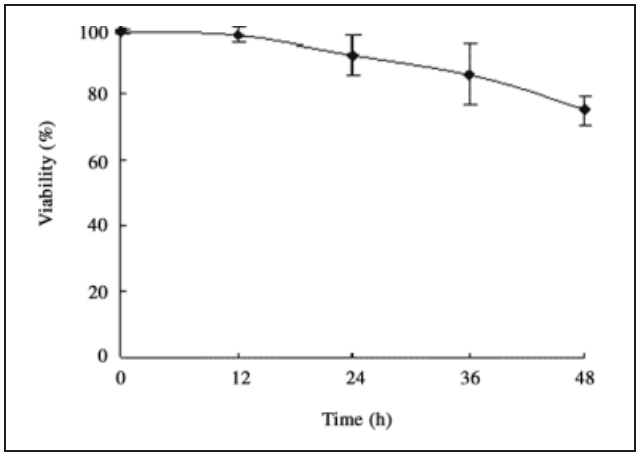


Fig. 7: Viability of HLECs as determined by trypan blue exclusion tests. Each point represents the mean ± S.D. of 3 experiments

2.2.5. RT-PCR and electrophoresis

There is a report about the mechanism of DSF-DDC conversion by ALDH (E.C.1.2.1.3) (Vallari and Pietruszko 1982). In human cornea and lens, ALDHs exhibit very high levels of activity (King and Holmes 1998). Therefore, we presume DSF-DDC conversion to happen on cultured HCECs monolayer by action of ALDHs. The assumption was approved here by the results of the drug penetration experiment. In addition, the expression of ALDH3a1 in HCECs is shown in Fig. 8.

However, there seems no obvious relationship in quantity between the amount of ALDH3a1 expression and the accumulated drug concentration in a proper penetration time (data not shown). It was reported that ALDH3 and ALDH1 represent approximately 5% and 3% of soluble corneal protein in soluble extracts of the human cornea, and these enzymes are predominantly restricted in the distribution to the epithelial and endothelial cells of the cornea (King and Holmes 1998). Hence, besides ALDH3, ALDH1 may play a role in DSF-DDC conversion as well.

Compared with the results of DSF penetration through HCECs and isolated rabbit cornea, none but DDC was found during DSF penetrating through the isolated rabbit cornea, while both DDC and DSF were found during DSF penetration through HCECs. As mentioned above, ALDHs also express in endothelial cells, so we estimate that in the endothelial layer DSF-DDC conversion might happen as well. In addition, except for ALDHs, whether other enzymes in human corneas take part in DSF-DDC conversion or not is still not clear. Further studies are needed to investigate the mechanism of DSF-DDC conversion in human eyes. Based on the results of our experiments by now, we suggest the process of DSF penetration through cornea as shown in the Scheme.

In conclusion, we carried out DSF and DDC *in vitro* ocular penetration experiments using the cultured immortalized HCECs, HLECs and isolated rabbit corneas. The results indicate that the *in vitro* cell culture of HCECs and HLECs may be effective to gain predictive ocular perme-

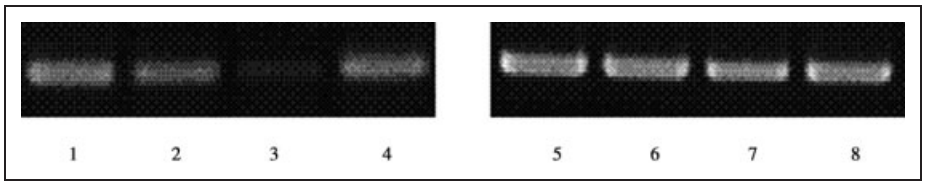
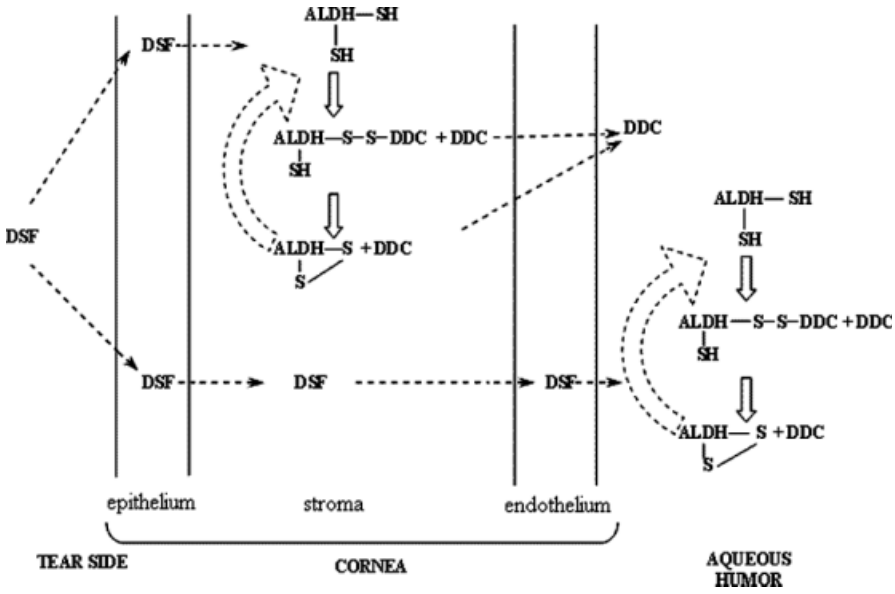


Fig. 8: Ethidium bromide stained agarose gel after RT-PCR amplification of GAPDH and ALDH3a1 mRNA expression in HCECs. lane 1: ALDH3a1 (4 days cell culture); lane 2: ALDH3a1 (7 days cell culture); lane 3: ALDH3a1 (14 days cell culture); lane 4: ALDH3a1 (21 days cell culture); lane 5: GAPDH (4 days cell culture); lane 6: GAPDH (7 days cell culture); lane 7: GAPDH (14 days cell culture); lane 8: GAPDH (21 days cell culture)

Scheme: Scheduled imagined process of DSF penetration through cornea



ability information while screening new formulations. It will be helpful to raise our experiment efficiency. The mechanism of DSF-DDC conversion in human corneas needs further investigation.

3. Experimental

3.1. Cell culture

SV-40 immortalized Human Cornea Epithelial Cells (HCECs) were cultured with Dulbecco's Modified Eagle's Medium (D-MEM)/Ham's F_{12} (GIBCO^{TM}, Tokyo, Japan), 1% streptomycin-penicillin (GIBCO^{TM}, Tokyo, Japan), and 5% fetal bovine serum (FBS). SRA01/04 cells (transformed human lens epithelial cells, HLECs) were cultured in D-MEM (GIBCO^{TM}, Tokyo, Japan) supplemented with 0.1% gentamicin (GIBCO^{TM}, Tokyo, Japan) and 10% fetal bovine serum (JRH Bioscience, USA.). Cells were incubated under 5% CO_2 and 95% O_2 at 37 °C. The medium was changed every alternate day. During culture, cell morphology was observed by a phase-contrast microscope (OLYMPUS CKX41, Japan) and photographed (OLYMPUS DP12-2, Japan). When reaching the condition of 80% confluence, the cells were trypsinized with 0.05% trypsin-EDTA (GIBCO^{TM}, Tokyo, Japan), then the separated cells were seeded on 6-well Transwell-Clear Polyester Membrane Inserts (surface area 4.7 cm^2 and 0.4 μm pore size, Costar, MA) and allowed to form subcultures. The inserts were precoated with collagen type I from rat tail and collagen type IV from human placenta (Sigma-Aldrich, Japan) and the cells planting density was 9×10^4 cells/cm^2 and 2×10^4 cells/cm^2 for HCECs and HLECs respectively. HCECs reached confluence after 7 days culture. Then the cells were exposed to air-liquid interface for $2 \sim 3$ weeks.

3.2. Determination of transepithelial electrical resistance (TEER)

Final TEER across the cell monolayer was detected by chopstick electrodes connected to an epithelial voltohmmeter (Millicell-ERS, Millipore, USA). TEER determination was performed during cell culture on 6-well Transwell-Clear Polyester Membrane Inserts before and after the penetration experiment. Net TEER was obtained by subtracting TEER of the blank inserts precoated with collagen. TEER values were received by multiplication with the effective culture area of the inserts.

3.3. Permeation experiments with cultured cells

3.3.1. DSF penetration through HCECs monolayer

DSF penetration solution was prepared with DSF/hydroxypropyl-β-cyclodextrin (HP-β-CD) (containing 1.26% HP-β-CD, 1 mg/ml DSF and 0.01% HPMC). The permeation experiment (Ouchi Shinko, Japan) was carried out by adding 2.5 ml Hank's balanced salt solution (HBSS, GIBCO^{TM}, Japan) with 25 mmol/l HEPES (Dojindo, Japan) (pH 7.4) to the basolateral side and 1.5 ml penetration solution to the apical side. At 15 min, 30 min, 45 min, 60 min, 90 min and 120 min, aliquots of 10 μl were taken from the basolateral side and replaced with an equal volume of HBSS with 25 mmol/l HEPES (pH 7.4). The samples were stored in $0\,°C \sim 4\,°C$ refrigerator immediately after taking to avoid drug degradation. The content of drug was analyzed by HPLC (Wang et al. 2004). Data analysis was performed as reported by the reference (Toropainen et al. 2001). Briefly, apparent permeability coefficient of the cultured monolayer and filter together were calculated according to the following equation:

$$P_{app} = V(dC/dt)/AC_0 \quad (1)$$

Where P_{app} is the apparent permeability coefficient of drug through cell monolayer, dC/dt is the drug flux across the membrane, V (cm^3) is the volume of the basolateral compartment, A (cm^2) is the surface area of the culture model and C_0 (mmol/l) is the initial drug concentration in the apical compartment. The experiment was performed in triplicate and expressed as mean ± S.D. values.

3.3.2. DDC penetration through HLECs monolayer

DDC (Wako Pure, Japan) was dissolved in HBSS with 25 mmol/l HEPES (pH 7.4) to the concentration of 0.1% (w/v) as penetration solution. DDC penetration experiment and data analysis was performed according to the same procedure as with DSF. The content of DDC was analyzed by HPLC (Wang et al. 2004).

3.3.3. Permeability studies with excised cornea

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research. The experiment was undertaken with the method reported (Wang et al. 2004). Briefly, Male rabbits (weight $2.5 \sim 3.0$ kg) were killed by a marginal ear-vein injection of a lethal dose of pentobarbital. The eyes were extirpated and the corneas were carefully separated from other ocular tissues. Then, the separated corneas were mounted in a methacrylate cell designed for trans-corneal transit experiments. The side of the chamber (donor) with the exterior surface of the cornea was filled with DSF penetration solution. The other side of the chamber (acceptor) contained HBSS with 25 mmol/l HEPES (pH 7.4). The transit experiments lasted for 2 h at 35 °C. 50 μl the sample solution were withdrawn from the acceptor at the indicated time. The concentration of DDC in the sample was determined as described above. The viability of the cornea was monitored by measuring its thickness and wet weight (no significant changes in thickness or weight over 2 h).

3.4. Trypan blue exclusion assay

Trypan blue dye exclusion was used to measure the extent of cytotoxity of DDC solution against HLECs monolayer. HLECs were seeded in the culture dish precoated with collagen type IV. After 4 days culture, cells were treated with the concentration of DDC solution 5.0 mmol/ml at different time intervals 12 h, 24 h, 36 h and 48 h respectively. Then cells were collected and cell suspension was diluted with 0.4% trypan blue solution (GIBCOBRL®, Life Technologies) in PBS (0.01 mmol/l) (GIBCO^{TM}, Japan). Cells were counted by a haemocytometer under a microscope. The percentage of viability was calculated as the number of cells unstained with trypan blue/ the number of total cells × 100%. Viability of cells before and after the penetration experiment with the cell culture model was determined by trypan blue exclusion assay as well.

3.5. RT-PCR and electrophoresis

HCECs were cultured on cell culture dishes (Corning, 60 mm dish style) in the presence or absence of collagen type I precoating. When cells were confluent, total RNA was isolated from HCECs by using RNeasy Minikit(50) reagent according to the manufacture's instruction and was used to measure the absorbance at 260 nm (nucleic acids) and 280 nm (protein), where the value of absorbance at 260 nm equals 40 μg RNA per milliliter. The final reaction volume of RT was 10 μl with 0.5 μg extracted RNA. Reaction components: 5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3). 10 units RNase inhibitor, 1.0 mmol/l deoxynucleotide triphosphates, 2.5 units avian myeloblastosis virus reverse transcriptase, 1.25 pmol oligo dT-M4 adapter primer, 5′-GTTTTCCCAGTCACGAT_{20}-3′ (Takara, Japan). The reaction tubes were incubated at 42 °C for 30 min, subsequently, 99 °C for 5 min, finally, 5 °C for 5 min. PCR was conducted as follows: Total RT reaction product was mixed with 10 μl 5 × PCR buffer, 1.25 units *Taq* polymerase (Takara, Japan), 0.5 μmol/l primers of ALDH3A1-F: 5′-AGAGGTTCGACCATATCCTGTA-3′ and ALDH3A1-R: 5′-TGATTCTTCCATAGTCCCGGGA-3′ for $ALDH3a_1$, 5′-GGT GCTGAGTATGTCGTGGAGTCTAC-3′ and 5′-CATGTAGGCCATGAGGTCCACCACC-3′ for glyceraldehydes-3-phosophate dehydrogenase (GAPDH) primers respectively to the final volume (50 μl). PCR was started at 94 °C for 2 min, followed by 30 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (60 s), and then 72 °C for 10 min, finally 5 °C for 10 min. We analyzed the amplification products by 1.5% agarose gels electrophoresis with ethidium bromide as staining reagent. Thereafter, ethidium bromide staining of the bands was photographed using CCD camera system, Imagemaster-CL (Amersham Biosciences, Piscataway, NJ, USA). Density analysis was performed using NIH Image (Scion Corp, USA).

3.6. Statistical analysis

All values were expressed as the means ± S.D. in a total of 3 to 5 experiments. Significance of the mean differences in each experiment was analyzed by Student's t-test and a p value of <0.05 was considered significant.

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