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Fabrication of a Corneal Model Composed of Corneal Epithelial and Endothelial Cells via a Collagen Vitrigel Membrane Functioned as an Acellular Stroma and Its Application to the Corneal Permeability Test of Chemicals

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ABSTRACT

A collagen vitrigel membrane (CVM) we developed can function as both a scaffold for cells and a pathway for chemicals. To extrapolate the corneal permeability of chemicals in vivo, we proposed six corneal models using the CVM. Thin and thick CVMs were used as models for Bowman's membrane (BM) and an acellular stroma (AS), respectively. Models for a corneal epithelium (CEpi), a CEpi-AS, a CEpi-endothelium (Endo), and a CEpi-AS-Endo were fabricated by culturing corneal epithelial cells and/or corneal endothelial cells on the surface of CVMs. Subsequently, the permeability coefficient (P_{app}) value of each model was calculated using five chemicals with different molecular radii; cyanocobalamin and four fluorescein isothiocyanate-dextrans (FD) (FD-4, FD-10, FD-20, and FD-40). The slopes of P_{app} versus molecular radii of those chemicals in the both BM and AS models were almost similar to data using an excised rabbit corneal stroma. The ratios of P_{app} values in models for BM, CEpi, and CEpi-Endo against those in data using an excised rabbit cornea were calculated as 75.4-fold, 6.4-fold, and 4.5-fold for FD-4, and 38.7-fold, 10.0-fold, and 4.2-fold for FD-10, respectively. Similarly, those in models for AS, CEpi-AS, and CEpi-AS-Endo were calculated as 26.1-fold, 2.5-fold, and 0.6-fold for FD-4, and 26.1-fold, 1.5-fold, and 0.6-fold for FD-10, respectively. These results suggest that the CEpi-AS-Endo model with both the barrier function of corneal cell layers and the diffusion capacity of chemicals in thick CVM is most appropriate for extrapolating the corneal permeability of chemicals in vivo.

Introduction

Corneal permeation studies required for ophthalmic drug development are generally performed by the test methods using laboratory animals in vivo or ex vivo (Hahne et al., 2012; Dave et al., 2015). However, animal experiments have disadvantages such as ethical issues on the sacrifice of life, high costs, poor reproducibility, and the questionable extrapolation of animal results to humans (Acheampong et al., 2002; Reichl and Becker, 2008; Scott et al., 2010; Baranowski et al., 2014; Pescina et al., 2015). To overcome these issues on animal experiments, a novel corneal model in cell culture systems in vitro appropriate for extrapolating the corneal permeability of chemicals in vivo is required for promoting the efficient development of eye drops.

The human cornea is made of three different tissues. The corneal epithelium (CEpi), stroma, and endothelium (Endo), in order from the outside, are formed by approximately six corneal epithelial cell layers with barrier function, keratocytes scattered in high-density collagen fibrillar layers approximately 500 μm thick with diffusional inhibition, and an endothelial cell monolayer with barrier function, respectively. The corneal permeability of chemicals is mainly dependent upon the barrier function of CEpi and is collaterally regulated by the diffusion rate of corneal stroma and the barrier function of corneal the Endo (Reichl

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ABBREVIATIONS: AS, acellular stroma; BM, Bowman's membrane; CEpi, corneal epithelium; CVM, collagen vitrigel membrane; CXM, collagen xerogel membrane; DF-medium, 1:1 mixture medium of Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 supplemented with 5% heat-inactivated fetal bovine serum, 5 μg/ml recombinant human insulin, 10 ng/ml recombinant human epidermal growth factor, 0.5% dimethyl sulfoxide, 100 U/ml penicillin, and 100 μg/ml streptomycin; D-medium, Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin; Endo, endothelium; FD, fluorescein isothiocyanate-dextran; HBSS, Hank's balanced salt solution; MUC1, Mucin 1, cell surface associated; O.C.T., Optimal Cutting Temperature; P_{app}, permeability coefficient; PBS, phosphate-buffered saline; PET, polyethylene terephthalate; TEER-end, transendothelial electrical resistance; ZO-1, zonula occludens-1.

and Becker, 2008). Therefore, the single models of CEpi and the combined models of CEpi-stroma-Endo in cell culture systems have been developed by using various scaffolds. CEpi models exhibited multilayer architecture like CEpi in vivo and revealed the formation of epithelial barrier function with rising transepithelial electrical resistance values. However, the CEpi models lack the effect of corneal stroma and Endo. From this viewpoint, several corneal models composed of not only the epithelium but also the stroma and Endo have been developed by using three different types of corneal cells, and unique scaffolds functioned as an artificial corneal stroma (e.g., crosslinked collagen-chondroitin sulfate) (Griffith et al., 1999), an acellular corneal matrix derived from porcine (Xu et al., 2008), and a type I collagen gel (Reichl et al., 2004). Such corneal models in vitro well mimicked cornea in vivo in morphologic and physiologic features. Especially, the corneal model using the collagen gel showed the similar behavior with excised porcine cornea in the permeability of model drugs such as pilocarpine hydrochloride, befunolol hydrochloride, and hydrocortisone. However, none of the corneal models in vitro have been in widespread use as pharmaceutical routine works. One of the reasons is the limitation of producing the same models due to disadvantages of previous scaffolds, such as mechanical weakness, variation of animal-derived materials, and complex preparation. Therefore, the novel scaffold that allows the brief and reproducible fabrication of a corneal model is necessary for promoting the efficient development of eye drops.

A collagen vitrigel membrane (CVM) is a stable gel produced by rehydration after vitrification of a traditional collagen gel, and consequently it is formed of high-density collagen fibrils, which are comparable to connective tissues in vivo. The CVM is tough and transparent, and bioactive chemicals with various molecular weights can penetrate it. Therefore, it is easily handled with forceps and functions as a scaffold to reconstruct useful tissues for the studies on, for example, regenerative medicine, drug developments, and alternatives to experimental animals. (Takezawa et al., 2004, 2007a,b,c). Moreover, we developed a mass fabrication technology for not only a thin CVM but also a thick CVM (Takezawa et al., 2012). We established a fabrication method for a CEpi model using an air-liquid interface culture system. The culture system facilitates the induction of layering corneal epithelial cells cultured on the CVM scaffold that was prepared on a polyethylene terephthalate (PET) membrane of a Millicell chamber (Takezawa et al., 2008, 2011). However, this model is unsuitable for immunohistological analyses by freeze sectioning due to the existence of hard PET membrane. Therefore, we developed a new chamber merely accompanying a CVM without the PET membrane and established its mass production process (Takezawa et al., 2012).

From the viewpoint of developing an ideal scaffold that functions as an artificial corneal stroma, the CVM has several advantages, such as mechanical toughness, reproducibility, and brief preparation. Moreover, the CVM is composed of 10%–25% collagen fibrils, equivalent to corneal stroma (Takezawa et al., 2007c; Reichl and Becker, 2008). In this study, we aimed to define an optimum corneal model involving a CVM scaffold appropriate for extrapolating the corneal permeability of chemicals in vivo. CVM scaffolds with two different thicknesses of about 20 μm as a replacement for Bowman's membrane (BM) and of about 450 μm as an acellular corneal stroma were designed, and subsequently six corneal models composed of merely a thin CVM, CEpi on a thin CVM, CEpi-Endo via a thin CVM, merely a thick CVM, CEpi on a thick CVM, and CEpi-Endo via a thick CVM were fabricated (Fig. 1). Then, the permeability coefficient of each model was calculated by using five model chemicals with different molecular radii and was compared with that of excised rabbit cornea.

Materials and Methods

Antibodies and Reagents. Rabbit polyclonal antibodies for zonula occludens-1 (ZO-1), occludin, and connexin-43 were purchased from Thermo Fisher Scientific (Grand Island, NY), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Sigma-Aldrich (St Louis, MO), respectively. Mouse monoclonal antibodies for cytokeratin 3; Mucin 1, cell surface associated (MUC1); and Na^+-K^+ ATPase

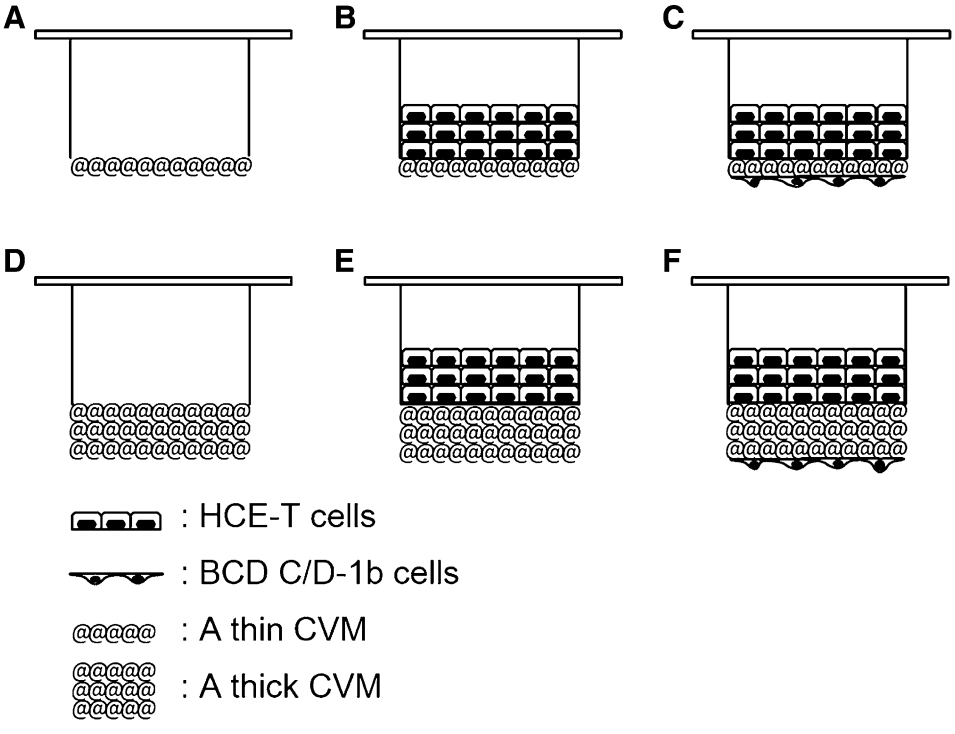

Fig. 1. Schematic illustration of six corneal models using chambers with thin and thick CVM scaffolds. BM model (A), CEpi model (B), CEpi-Endo model (C), AS model (D), CEpi-AS model (E), and CEpi-AS-Endo model (F). Black lines represent plastic cylinders and hangers of a CVM chamber.

were purchased from Progen Biotechnik GmbH (Heidelberg, Germany), Sanbio B.V. (Uden, Netherlands), and Merck Millipore (Darmstadt, Germany), respectively. A goat Alexa Fluor 555–conjugated secondary antibody for rabbit IgG and a goat Alexa Fluor 488–conjugated secondary antibody for mouse IgG were purchased from Thermo Fisher Scientific. Hoechst 33342 was purchased from Dojindo Laboratories (Kumamoto, Japan). Normal goat serum was purchased from Sigma-Aldrich. Tissue-Tek Optimal Cutting Temperature (O.C.T.) Compound was purchased from Sakura Finetek Japan (Tokyo, Japan). Cyanocobalamin and fluorescein isothiocyanate–labeled dextran (FD) with average molecular weights of 4, 10, 20, and 40 kDa (FD-4, FD-10, FD-20, and FD-40) were purchased from Sigma-Aldrich.

Preparation of CVM Chambers. A chamber (ad-MED Vitrigel) with a collagen xerogel membrane (CXM) containing 0.05 mg of type I collagen/1.0 cm^2 was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), which manufactured CXM chambers in accordance with our previous report (Takezawa et al., 2012). The CXM chamber was set in the well of a 12-well plate. Then, the CXM was immersed in a culture medium by pouring 1.5 ml outside and 0.5 ml inside the chamber in the well for 10 minutes to convert the xerogel into a vitrigel immediately before use, resulting in the preparation of a chamber with a thin CVM with a thickness of about 20 μm. Here, the thin CVM in the chamber can be used as a BM model because of the similarity of architecture and components. Regarding the chamber with a thick CVM with a thickness of about 450 μm [i.e., an acellular stroma (AS) model], we custom ordered a chamber with a CXM containing 1.125 mg of type I collagen/1.0 cm^2 from Kanto chemical Co., Inc. in a procedure similar to that for ad-MED Vitrigel.

Culture of HCE-T Cells and BCD C/D-1b Cells. A simian virus 40 immortalized human corneal epithelial cell strain (HCE-T cells; RCB #2280) was obtained from RIKEN BioResource Center (Tsukuba, Japan) (Araki-Sasaki et al., 1995). The cells were cultured and maintained in a 1:1 mixture medium of Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 supplemented with 5% heat-inactivated fetal bovine serum, 5 μg/ml recombinant human insulin, 10 ng/ml recombinant human epidermal growth factor, 0.5% dimethylsulfoxide, 100 U/ml penicillin, and 100 μg/ml streptomycin (DF-medium) at 37°C in a humidified atmosphere of 5% CO_2 in air.

An immortalized bovine corneal endothelial cell line (BCD C/D-1b cells; CRL-2048) was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured and maintained in a Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (D-medium) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Isolation and Characterization of an Endothelial Cell-Like Clone from BCD C/D-1b Cells. BCD C/D-1b cells suspended in the D-medium were seeded and cultured in a 96-well cell culture plate at a density of 0.5 cells/well. Several endothelial cell–like clones were morphologically selected by phase-contrast microscopy. Each clone was expanded to about 1×10^7 cells and preserved in liquid nitrogen. One clone representing excellent proliferative performance was subjected to the following experiment. The clonal cells recovered from liquid nitrogen were defined as passage 1 and were subcultured until passage 28 to estimate their morphologic stability.

To confirm the properties essential for the fabrication of a corneal endothelial layer, the endothelial cell–like clone was cultured in the chamber with a thin CVM and subjected to the time-dependent analyses for cell morphology, protein expression, and barrier function. The clonal cells suspended in 0.5 ml of the D-medium at a density of 2.0×10^5 cells/ml were seeded onto the thin CVM in a chamber preset in 1 well of a 12-well plate with 1.5 ml of the fresh medium in the well and cultured for 7 days. The media were changed at days 1, 4, and 7. The cell morphology and immunohistology for ZO-1 and Na^+-K^+ ATPase protein expressions were observed at days 1, 3, and 7 using a phase-contrast microscope (model TE300; Nikon, Tokyo, Japan) and a laser-scanning confocal microscope (model FV1000; Olympus, Tokyo, Japan), respectively. Also, the barrier function was analyzed by measuring the electrical resistance values of each CVM chamber before (R_{blank}) and after (R_{sample}) culturing the cells at days 0 (2 hours), 1, 2, 3, 4, and 7 using an electrical resistance meter (Kanto Chemical Co., Inc.). Transendothelial electrical resistance (TEER-end) value was calculated by using the following formula:

$$\text{TEER-end} = (R_{sample} - R_{blank}) \times \text{effective surface area}.$$

Here, the effective surface area of the CVM chamber was 1.0 cm^2.

Fabrication of Six Corneal Models. We prepared six corneal models, as shown in Fig. 1. The preparation of the BM model and AS model were described above. The CEpi model, CEpi-Endo model, CEpi-AS model, and CEpi-AS-Endo model were fabricated as follows.

In the fabrication of the CEpi model, HCE-T cells suspended in 0.5 ml of the DF-medium at a density of 1.2×10^5 cells/ml were seeded onto the thin CVM (i.e., a BM model) of a chamber preset in 1 well of a 12-well plate with 1.5 ml of the fresh medium in the well, and the cells were cultured for 2 days. Subsequently, the exterior medium in the chamber was changed and the interior medium in the chamber was removed to start the additional culture under the air-liquid interface for 4 days. The exterior medium of the chamber was changed on the third day in the additional culture. Also, the fabrication of the CEpi-AS model was achieved by using the thick CVM (i.e., an AS model) of the chamber in the above cell-seeding process.

In the fabrication of the CEpi-Endo model, HCE-T cells were first cultured for 3 days using the same procedure for fabricating the CEpi model. Subsequently, the CVM chamber was transferred to one well of a six-well plate and flipped upside down to make a reverse-side compartment for cell culture by fixing a plastic cylinder with an appropriate size (e.g., inner-outer diameter 11–16 mm; length 8.5 mm) onto the bottom of the CVM chamber. The endothelial cell–like clonal cells derived from BCD C/D-1b cells suspended in 0.5 ml of the D-medium at a density of 2.0×10^5 cells/ml were seeded into the reverse-side compartment. The clonal cells were cultured for 2 hours to induce sufficient adhesion to the CVM, resulting in the coculture with HCE-T cells via the CVM. Subsequently, the endothelial cell medium was removed and the cylinder was detached from the CVM chamber. Then, the CVM chamber was set in a well of a 12-well plate with 1.5 ml of the DF-medium, and both cells were cocultured for an additional 3 days under the air-liquid interface. Also, the fabrication of the CEpi-AS-Endo model was achieved by using the thick CVM (i.e., an AS model) in the chamber in the above cell-seeding process.

All models were subjected to histology and permeability examinations.

Histology and Immunohistology. For clarifying morphologic characteristics, corneal endothelial layers in a CVM chamber at days 1, 3, and 7 and three corneal models (CEpi-Endo model, CEpi-AS model, and CEpi-AS-Endo model) except for the CEpi model, as previously reported (Yamaguchi et al., 2013) were isolated from the plastic cylinder of the CVM chamber using an appropriate disposable biopsy punch and fixed for 5 minutes in methanol kept on ice immediately after sufficiently chilling it at −45°C.

The fixed corneal endothelial layers were washed with phosphate-buffered saline (PBS) three times. Subsequently, they were incubated with PBS containing 1% normal goat serum for 30 minutes to block nonspecific adsorption of antibodies. Then, the first antibodies against ZO-1 or Na^+-K^+ ATPase prepared in PBS containing 1% normal goat serum at a concentration of 5 μg/ml were applied and incubated for 16 hours at 4°C, followed by washing with PBS three times. The secondary antibodies against rabbit IgG or mouse IgG prepared in PBS containing 1% normal goat serum at a concentration of 4 μg/ml were applied and incubated for 3 hours at room temperature, followed by washing with PBS three times. Subsequently, cell nuclei were counterstained with Hoechst 33342. Stained cells were observed by a laser-scanning confocal microscope (model FV1000; Olympus).

The fixed corneal models were embedded in an O.C.T. compound after removing the excessive methanol around them with an absorbent paper towel, frozen in liquid nitrogen, and stored at −80°C. The samples were vertically cut into cross sections with a thickness of 5 μm against the CVM using a cryostat (model CM3050S; Leica Microsystems, Wetzlar, Germany). The frozen sections spread on a glass slide were dried out for 60 minutes at room temperature. For histology, the sections were immersed in water for 5 minutes to remove the O.C.T. compound, stained with hematoxylin and eosin, and observed by a light microscope (model E600; Nikon). For immunohistology, the sections derived from the CEpi-AS model were subjected to immunostaining using the first antibodies against ZO-1, occludin, connexin-43, cytokeratin 3, or MUC1, and to counterstaining using Hoechst 33342 in a similar procedure, as mentioned above.

Calculation of Permeability Coefficient. All six corneal models were subjected to the permeability test using the following five test chemicals: cyanocobalamin, FD-4, FD-10, FD-20, and FD-40, representing approximately 8.5, 14, 23, 33, and 45 Å in Stokes' radius, respectively. Also, a commercially available Millicell hanging cell culture insert possessing a multiporous PET membrane with a pore size of 1.0 μm (MCRP12H48; Merck Millipore, Burlington, MA) were subjected to the permeability test as a control chamber with a common scaffold for fabricating corneal models. Each corneal model and Millicell control were set in the well of 12-well plate, and subsequently 1.8 and 0.5 ml of Hanks' balanced salt solution (HBSS) were poured into the outside and inside of the chamber, respectively. After incubating the plate for 10 minutes at 37°C, the inside

HBSS of each chamber was changed to the test chemical solution, the same volume of HBSS containing 10 μg/ml cyanocobalamin, 5 μg/ml FD-4, 5 μg/ml FD-10, 5 μg/ml FD-20, or 5 μg/ml FD-40. To quantitatively analyze the time-dependent penetration of test chemicals from the inside to the outside of the chamber, 100 μl of HBSS in the outside of the chamber was sampled and the same volume of fresh HBSS was returned there at every 10 minutes from the starting point immediately after adding the test chemical solution to the ending point, with an elapsed time of 180 minutes. The concentration of cyanocobalamin or FDs in each sample was analyzed by measuring the absorbance at 365 nm using an absorbance multiplate reader (VersaMax Microplate Reader; Molecular Devices, Sunnyvale, CA) or the fluorescent intensity at 490 nm (excitation) and 520 nm (emission) using a fluorescence multiplate reader (SpectraMax Gemini XS Microplate Reader; Molecular Devices), respectively.

The permeability coefficients of test chemicals in each model were calculated using the following formula:

$$P_{app} = dQ/dT \times 1/A \times 1/C_0.$$

Here, P_{app}, dQ/dT, A, and C_0 represent the permeability coefficient (centimeters per second), the flux (micrograms per second), the surface area of each model (square centimeter) and the initial chemical concentration (micrograms per milliliter), respectively.

The P_{app} values of FD-4 and FD-10 in excised male rabbit corneas are reported as 0.056×10^{-6} and 0.031×10^{-6} cm/s, respectively (Sasaki et al., 1995). The ratios of P_{app} values for FD-4 and FD-10 in all six corneal models against those in the report using excised rabbit corneas were calculated by dividing each P_{app} value of the former by that of the latter. Here, the ratio of 1.0 represents an ideal model equivalent for excised rabbit cornea. Next, the contribution of epithelium and Endo in six corneal models were analyzed by the concept for reduction degree when the P_{app} values of CEpi (or CEpi-AS) models were compared with those of BM (or AS) models, and when the P_{app} values of CEpi-Endo (or CEpi-AS-Endo) models were compared with those of CEpi (or CEpi-AS) models, respectively. Here, the reduction degree for the epithelium was calculated using the following formula:

$$\text{Reduction degree for epithelium (\%)} = \{1 - (P_{app} \text{ of CEpi model})/(P_{app} \text{ of BM model})\} \times 100 \text{ or } \{1 - (P_{app} \text{ of CEpi-AS model})/(P_{app} \text{ of AS model})\} \times 100$$

Similarly, the reduction degree for the Endo was calculated using the following formula:

$$\text{Reduction degree for endothelium (\%)} = \{1 - (P_{app} \text{ of CEpi-Endo model})/(P_{app} \text{ of CEpi model})\} \times 100 \text{ or } \{1 - (P_{app} \text{ of CEpi-AS-Endo model})/(P_{app} \text{ of CEpi-AS model})\} \times 100$$

Results

Isolation and Characterization of an Endothelial Cell-Like Clone from BCD C/D-1b Cells. BCD C/D-1b cells before cloning grew and formed a monolayer in a culture dish. However, the individual cellular shapes were not uniform polygons but were of different varieties such as small round, cobblestone, spindle, and hypertrophy. Also, some interstices were observed especially around the fibroblast-like cells even in the confluent stage (Fig. 2A). Several endothelial cell–like clones showing the morphology of a cobblestone were successfully isolated from the BCD C/D-1b cells by the limiting dilution method. One clone representing excellent proliferative performance was subjected to the following experiment. The endothelial cell–like clonal cells at passage 1 formed a confluent monolayer and the individual cellular shapes represented cobblestones (Fig. 2B). Also, the clonal cells at passage 28 showed the same morphologic properties as that at passage 1 (Fig. 2C), suggesting that the clonal cells could maintain the morphologic stability for the following experimental period.

The clonal cells cultured in a CVM chamber for 1 day formed a confluent monolayer with loose cell-to-cell communications in which the individual cellular shapes represented nonuniform cobblestones (Fig. 3A). The cells expressed Na^+-K^+ ATPase, which is an active transporter that is known as a marker of corneal Endo (Fig. 3D); however, they rarely expressed ZO-1, which is a tight junction-associated protein (Fig. 3G). Meanwhile, the cells on day 3 fabricated a confluent monolayer with tight cell-to-cell communications in which the individual cellular shapes represented hexagons, and they well expressed not only Na^+-K^+ ATPase, but also ZO-1 (Fig. 3, B, E, and H). Subsequently, the cells on day 7 showed the more uniform thickness of a corneal endothelial layer with expressions of Na^+-K^+ ATPase and ZO-1 (Fig. 3, C, F, and I).

Also, the TEER-end values of the clonal cells cultured in a CVM chamber increased for the initial 3 days with significant differences, and subsequently values were nearly constant without significant differences until day 7 (Fig. 4).

These data suggest that a corneal endothelial layer with barrier function was fabricated by culturing the clonal cells in a CVM chamber for 3 days.

Histological and Immunohistological Characterization of Corneal Models. Histological and immunohistological characteristics of four corneal models (CEpi model, CEpi-AS model, CEpi-Endo model, and CEpi-AS-Endo model) were compared.

As previously reported (Yamaguchi et al., 2013), the CEpi model on a thin CVM possessed about six layers of HCE-T cells with barrier function showing the transepithelial electrical resistance value of about 60 Ω/cm^2 and resembled human CEpi in the expression pattern of five CEpi-associated proteins, ZO-1, occludin, connexin-43, cytokeratin 3, and MUC1. Also, the CEpi-AS model possessed about six layers of HCE-T cells on a thick CVM (Fig. 5A). The uppermost layer was covered with extremely flattened cells, and the other layers were mostly composed of squamous cells (Fig. 5B). Regarding the five CEpi-associated proteins, ZO-1, and occludin that are tight junction–associated proteins were abundantly expressed

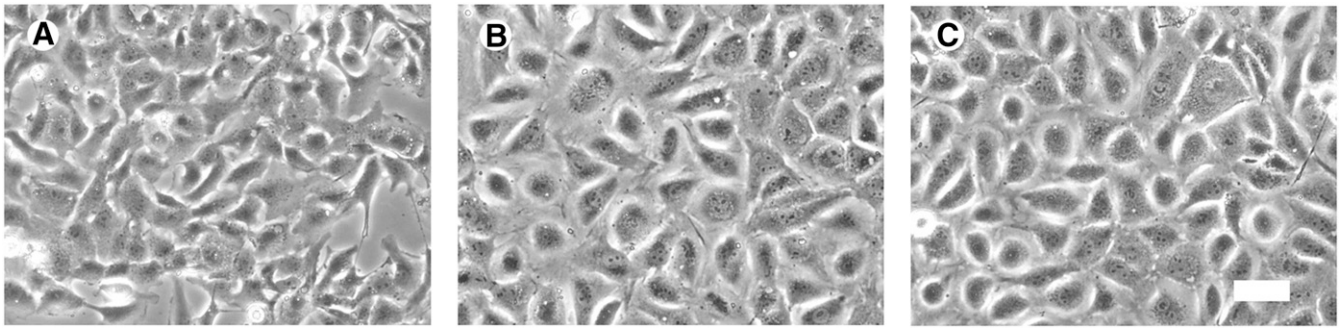

Fig. 2. Phase-contrast microscopic observations of BCD C/D-1b cells. BCD C/D-1b cells before cloning (A) and endothelial cell–like clonal cells at passage numbers 1 (B) and 28 (C) after cloning were cultured in a tissue culture plate. Scale bar, 50 μm.

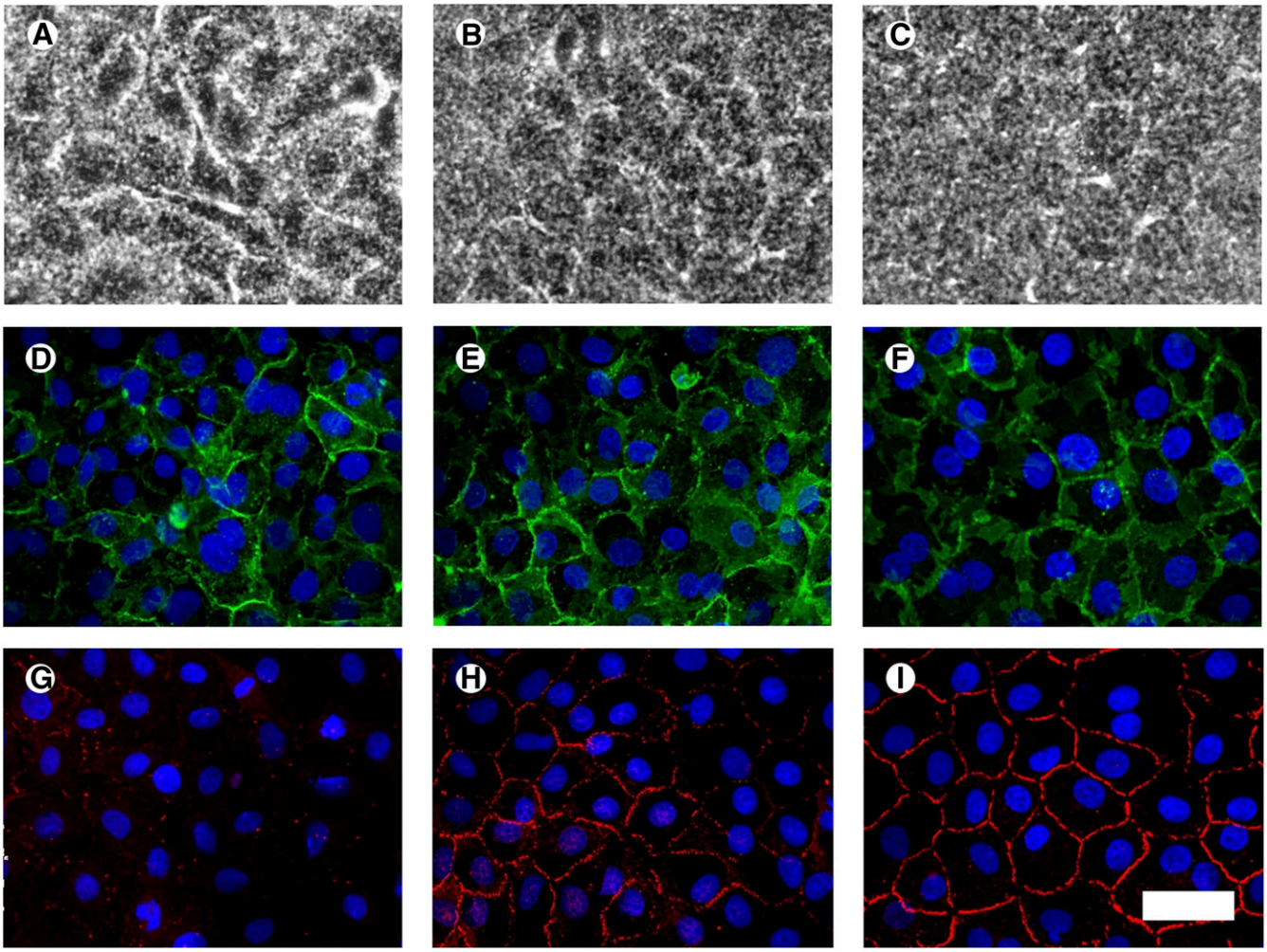

Fig. 3. Microscopic observations of a corneal endothelial layer in a CVM camber. The endothelial cell–like clonal cells from BCD C/D-1b cells were cultured for 1 day (A, D, and G), 3 days (B, E, and H), and 7 days (C, F, and I). The clonal cells were observed with a phase-contrast microscope (A–C), and were stained with antibodies for Na^+-K^+ ATPase (D–F) and ZO-1 (G–I). The nuclei of cells were counterstained with Hoechst 33342 (D–I). Scale bar, 50 μm.

in the lateral and basal surfaces of cells in the superficial layer in comparison with the other layers (Fig. 5, C and D). Connexin-43, consisting of gap junctions and cytokeratin 3, which is a type II cytokeratin in CEpi, were expressed in the membrane and cytoplasm of cells in all layers, respectively (Fig. 5, E and F). MUC1, which is a cell membrane–spanning mucin, was only expressed in the apical surface of cells in the superficial layer (Fig. 5G). These data demonstrated that the epithelial region of the CEpi-AS model is the same as the CEpi model and resembled human corneal epithelium in vivo.

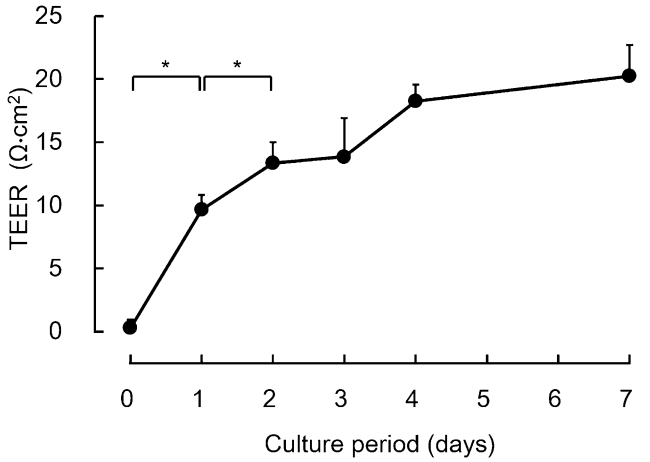

Fig. 4. Time-dependent change of TEER-end values of a corneal endothelial layer in a CVM camber. Each value represents the mean ± S.D. (n = 3). *$P < 0.05$.

The CEpi-Endo model and the CEpi-AS-Endo model exhibited architectures composed of about six cell layers of HCE-T cells and a monolayer of endothelial cell–like clonal cells derived from BCD C/D-1b cells via thin and thick CVMs, respectively (Fig. 6). The histological data demonstrated that the CEpi-AS-Endo model well reflected the vertical structure of the human cornea in vivo.

Permeability Coefficient of Test Chemicals in Corneal Models. The P_{app} values of cyanocobalamin, FD-4, FD-10, FD-20, and FD-40 in the six corneal models were calculated as shown in Table 1. The P_{app} values of cyanocobalamin in CEpi-Endo, CEpi-AS, and CEpi-AS-Endo models cannot be calculated because the concentrations in each sample were lower than the detection limit.

In the comparison of acellular models with a common scaffold for fabricating corneal models, the slopes of permeability coefficients versus the molecular radii of test chemicals in the BM model, AS model, and commercially available multiporous PET membrane were −2.64, −2.04 and −0.84, respectively (Fig. 7). These results reveal that the P_{app} value

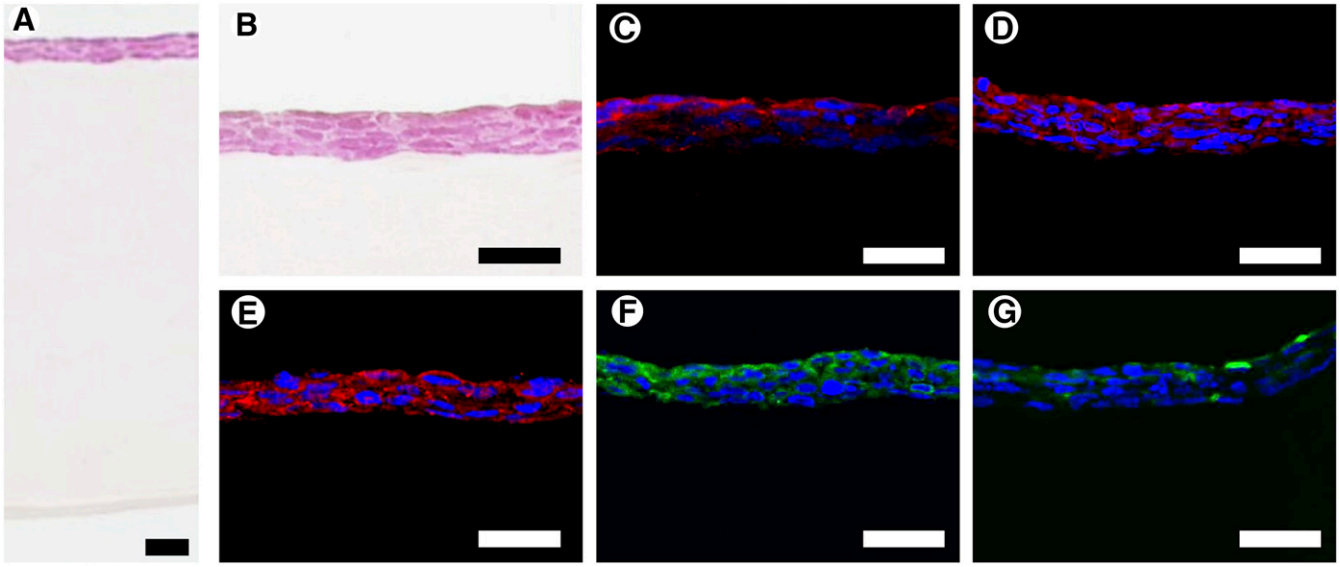

Fig. 5. Microscopic observations of a CEpi-AS model. Cross sections of the model were stained with hematoxylin and eosin (A and B) and were also stained with antibodies for ZO-1 (C), occludin (D), connexin-43 (E), cytokeratin 3 (F), and MUC1 (G). The nuclei of cells were counterstained with Hoechst 33342 (C–G). Scale bar, 50 μm.

corresponding to each test chemical in both models exponentially decreases as its molecular radius increases, although in the multiporous PET membrane it does slightly increase. In addition, the P_{app} values of cyanocobalamin, FD-4, FD-10, and FD-20 in the AS model were significantly lower compared with that in the BM model, suggesting that the permeability coefficient of test chemicals was regulated by the thickness of the CVM used for each model.

In the comparison of culture models with an excised rabbit cornea, the P_{app} values of FD-4 and FD-10 were reduced, in order, in the CEpi model, CEpi-Endo model, CEpi-AS model, and CEpi-AS-Endo model. Also, the ratios of P_{app} values in models for BM, CEpi, and CEpi-Endo to those in data using an excised rabbit cornea were calculated as 75.4-fold, 6.4-fold, and 4.5-fold for FD-4, and 38.7-fold, 10.0-fold, and 4.2-fold for FD-10, respectively. Similarly, the ratios of P_{app} values in models for AS, CEpi-AS, and CEpi-AS-Endo were calculated as 26.1-fold, 2.5-fold, and 0.6-fold for FD-4, and 26.1-fold, 1.5-fold, and 0.6-fold for FD-10, respectively. Meanwhile, the effects of epithelium and Endo on the degree of reduction of P_{app} values were 90.4% and 76.4% for FD-4, and 94.3% and 58.7% for FD-10, respectively. In particular, there was no significant difference between the P_{app} value of the CEpi-AS-Endo model and that of excised rabbit cornea (Fig. 8). These results reveal that the architecture composed of not only both epithelium and Endo but also AS is essential for fabricating a culture model equivalent to the excised rabbit cornea.

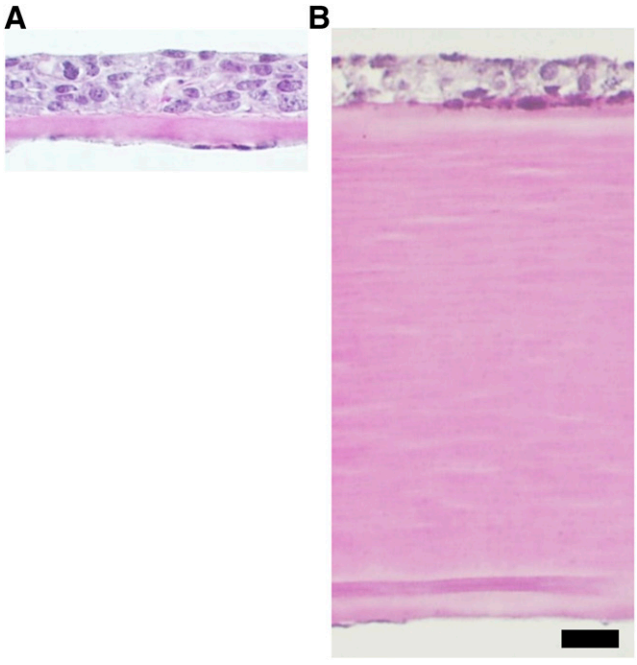

Fig. 6. Microscopic observations of corneal models composed of corneal epithelial cells and endothelial cells via a CVM. Cross sections of the CEpi-Endo model (A) and the CEpi-AS-Endo model (B) were stained with hematoxylin and eosin. Scale bar, 50 μm.

Discussion

The corneal permeability of drugs in vivo is mainly dependent upon the barrier function of epithelial cell layers on BM and is collaterally regulated by the diffusion capacity in stromal matrix and the barrier function of an endothelial layer on Descemet's membrane (Reichl and Becker, 2008). Here, the main component of BM, stromal matrix, and Descemet's membrane is a network of high-density collagen fibrils. In the current study, we focused on the network of high-density collagen fibrils functioned as not only a scaffold for cells but also a pathway for drugs and newly prepared a thick CVM equivalent to AS in addition to a previous thin CVM equivalent to BM. Based on this concept, six corneal models possessing matrix-dependent diffusion capacity and/or cell layer–dependent barrier function were fabricated by using thin and thick CVMs, corneal epithelial cells, and endothelial cells. The corneal models are BM model, CEpi model as previously described (Yamaguchi et al., 2013), CEpi-Endo model, AS model, CEpi-AS model, and CEpi-AS-Endo model. Subsequently, an optimal corneal model in vitro that is useful for extrapolating the corneal permeability was determined.

The CVM can play the role of not only a bifacial scaffold in three-dimensional cell culture, but also a pathway for proteins with a wide range of molecular weights (Takezawa et al., 2007a,b). From this viewpoint, we proposed six corneal models using chambers accompanying

TABLE 1

Permeability coefficient of six corneal models

Each value represents the mean ± S.D. of three independent experiments.

Type of Model		Permeability Coefficient				
		Cyanocobalamin	FD-4	FD-10	FD-20	FD-40
		$\times 10^{-6}$ cm/s				
Acellular model	BM	9.38 ± 0.47	4.22 ± 0.18	1.20 ± 0.31	0.46 ± 0.06	0.12 ± 0.002
	AS	4.45 ± 0.32	1.46 ± 0.14	0.81 ± 0.11	0.24 ± 0.03	0.17 ± 0.03
Culture model	CEpi	0.82 ± 0.097	0.36 ± 0.01	0.31 ± 0.02	0.36 ± 0.06	0.054 ± 0.01
	CEpi-Endo	Not detected	0.25 ± 0.04	0.13 ± 0.02	0.084 ± 0.01	0.050 ± 0.01
	CEpi-AS	Not detected	0.14 ± 0.06	0.046 ± 0.07	0.009 ± 0.002	0.017 ± 0.009
	CEpi-AS -Endo	Not detected	0.033 ± 0.02	0.019 ± 0.01	0.016 ± 0.005	0.033 ± 0.028

thin and thick CVMs (Fig. 1). The CVM potential as a scaffold for fabricating four culture models was examined as follows. First, we confirmed that the thin CVM provided an appropriate scaffold for corneal endothelial cell–like clonal cells, and consequently that a corneal Endo with barrier function and specific protein expressions was well reconstructed by culturing the clonal cells on it for at least 3 days (Figs. 2–4). Next, we fabricated four culture models as follows. A CEpi model with barrier function and specific protein expressions was prepared by culturing HCE-T cells on the thin CVM of the chamber for 6 days (data not shown), as previously reported (Yamaguchi et al., 2013). According to this fabrication procedure, a CEpi-AS model was prepared by culturing HCE-T cells on the thick CVM of the chamber for 6 days. Subsequently, the model was confirmed to exhibit both epithelial barrier function (data not shown) and specific protein expressions (Fig. 5). Also, a CEpi-Endo model and a CEpi-AS-Endo model were prepared by culturing HCE-T cells in the thin and thick CVM chambers for 2 days and subsequently coculturing corneal endothelial cell–like clonal cells on the opposite surface of CVMs for 4 days, respectively (Fig. 6). These data suggest that the CVM chambers in comparison with the traditional scaffolds are appropriate for mass production with high reproducibility and can provide easy handling in cell culture manipulation and short-term fabrication of not only tissue-type but also organ-type culture models.

The CVM potential as a pathway for penetrating five test chemicals was examined, as follows. We calculated the P_{app} values in both acellular models and the culture models from the viewpoint of the diffusion capacity in a network of high-density collagen fibrils and the barrier function of cell layer(s), respectively (Table 1). First, in the acellular models, the slopes of permeability coefficients versus molecular radii of test chemicals in the BM model, AS model, and commercially available multiporous PET membrane were investigated (Fig. 7). Meanwhile, the slope value in the excised rabbit corneal stroma was calculated as −2.52 from the previous report describing the P_{app} values of four test chemicals: phenylephrine, acebutolol, hemoglobin, and albumin representing approximately 4.0, 5.0, 31, and 35 Å in Stokes' radius, respectively (Prausnitz and Noonan, 1998). This work demonstrated that the P_{app} values decrease as the molecular radii increase in the excised rabbit corneal stroma. Also, our current data revealed that the P_{app} values decrease as the molecular radii increase in both a BM model and an AS model, and consequently their slope values were −2.64 and −2.04, respectively. Meanwhile, the slope of a multiporous PET membrane was −0.84. This calculation demonstrated that the slope in the excised rabbit corneal stroma almost matched both a BM model and an AS model, although it was far from that of a multiporous PET membrane. These findings suggest that acellular models using CVM chambers can be used as alternatives to the excised rabbit corneal stroma due to their similarity in the diffusion rate of chemicals. Next, in the culture models, we compared the P_{app} values of four culture models with that of an excised rabbit cornea to clarify the contribution of barrier function based on cell layers. Consequently, the ratios of P_{app} values of FD-4 in a BM model, a CEpi model, and a CEpi-Endo model against an excised rabbit cornea were calculated as 75.4-fold, 6.4-fold, and 4.5-fold, respectively. Also, those of FD-10 were calculated as 38.7-fold, 10.0-fold, and 4.2-fold, respectively. These calculations mean that the effect of the epithelium and Endo on the reduction degree of P_{app} values is 91.5% and 30.6% for FD-4, and 74.2% and 58.1% for FD-10, respectively. Similarly, those of FD-4 in an AS model, a CEpi-AS model, and a CEpi-AS-Endo model against an excised rabbit cornea were calculated 26.1-fold, 2.5-fold and 0.6-fold, respectively. Also, those of FD-10 were calculated as 26.1-fold, 1.5-fold and 0.6-fold, respectively. Meanwhile, the effect of the epithelium and Endo on the degree of reduction of P_{app} values is in the range of 74.2%–94.3% and

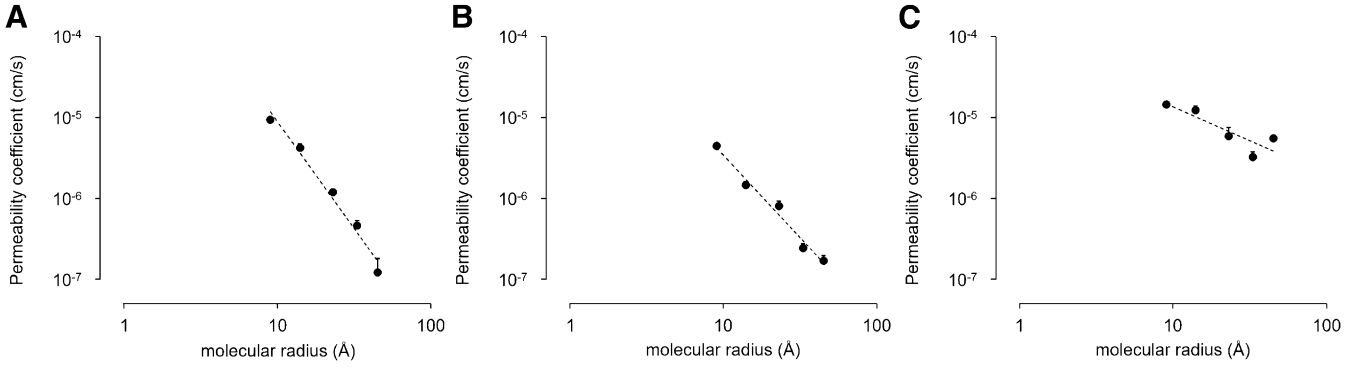

Fig. 7. Comparison among the permeability coefficient of a BM model (A), that of an AS model (B), and that of a commercially available multiporous PET membrane (C). Each permeability coefficient was tested using cyanocobalamin, FD4, FD10, FD20, and FD40. Dashed lines represent slopes calculated by least-squares test. Each value represents the mean ± S.D. (n = 3).

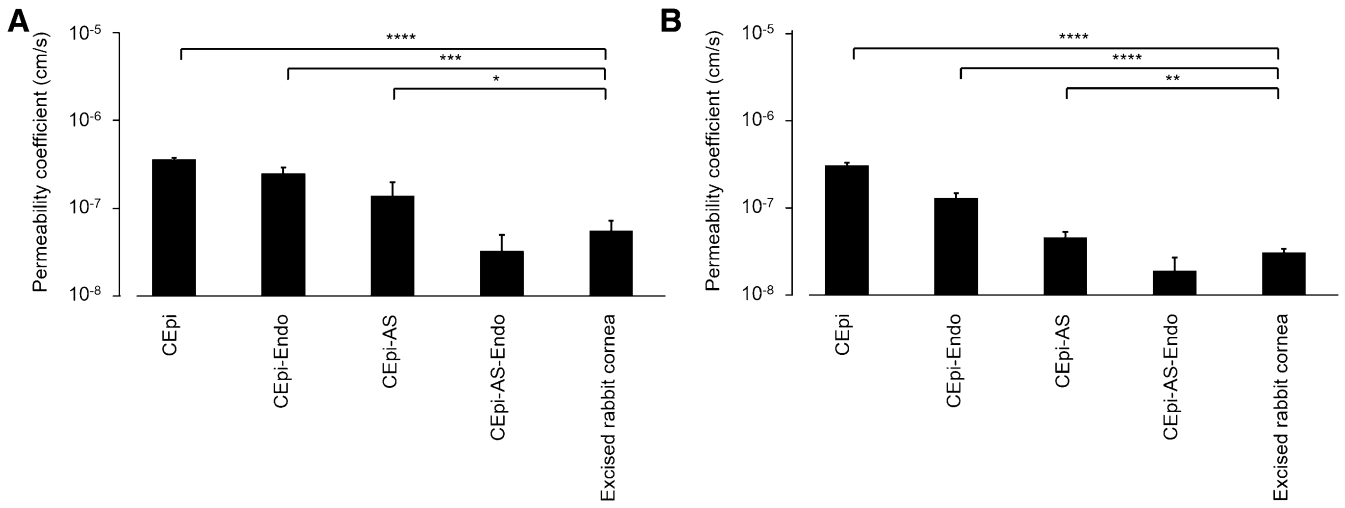

Fig. 8. Comparison between the permeability coefficient of FD-4 (A) and FD-10 (B) in each corneal model reconstructed in the CVM chamber described in Table 1 and that in an excised rabbit cornea. Each value represents the mean ± S.D. (n = 3). *$P < 0.1$; **$P < 0.05$; ***$P < 0.01$; ****$P < 0.001$.

30.6%–76.4%, respectively. This suggests that both epithelial and endothelial cell layers contribute to the regulation of the permeability coefficient, although the former effect is larger than the latter. Moreover, the P_{app} values of FD-4 and FD-10, not in a CEpi-Endo model but in a CEpi-AS-Endo model, were very close to those of an excised rabbit cornea (Fig. 8). These data suggest that not only the barrier function of both epithelial and endothelial cell layers but also the diffusion capacity in a network of high-density collagen fibrils is indispensable to fabricate corneal models in vitro that are appropriate for extrapolating the corneal permeability of chemicals in vivo. The advantages of our CEpi-AS-Endo models using a CVM scaffold are that the fabrication period is short and the handling in the permeability assay is easy compared with the traditional in vitro corneal models using a fragile scaffold (Reichl et al., 2004; Xu et al., 2008; Rönkkö et al., 2016). To investigate the feasibility of our CEpi-AS-Endo models in comparison with the traditional models in vitro, however, further studies using candidate chemicals for ophthalmic drugs are essential not only in the corneal permeability test but also in the metabolic assay.

In this study, we fabricated a novel corneal model using corneal epithelial cells, endothelial cells, and a CVM functioned as both a bifacial scaffold and a pathway for chemicals, and subsequently the permeability of test chemicals with different molecular sizes was analyzed to evaluate the correlation with the data on an excised rabbit cornea. Consequently, we succeeded in developing a new technology for extrapolating the corneal permeability of test chemicals. Therefore, we hope that this study provides a new concept for fabricating an artificial cornea as a research tool that will be useful for the development of ophthalmic drugs.

Authorship Contributions

Participated in research design: Takezawa.

Conducted experiments: Yamaguchi.

Performed data analysis: Yamaguchi, Takezawa.

Wrote or contributed to the writing of the manuscript: Yamaguchi, Takezawa.

References

Acheampong AA, Shackleton M, John B, Burke J, Wheeler L, and Tang-Liu D (2002) Distribution of brimonidine into anterior and posterior tissues of monkey, rabbit, and rat eyes. *Drug Metab Dispos* **30**:421–429.

Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, and Handa H (1995) An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci* **36**:614–621.

Baranowski P, Karolewicz B, Gajda M, and Pluta J (2014) Ophthalmic drug dosage forms: characterisation and research methods. *ScientificWorldJournal* **2014**:861904.

Dave V, Paliwal S, Yadav S, and Sharma S (2015) Effect of in vitro transcorneal approach of aceclofenac eye drops through excised goat, sheep, and buffalo corneas. *ScientificWorldJournal* **2015**:432376.

Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, Hakim M, Song Y, and Watsky MA (1999) Functional human corneal equivalents constructed from cell lines. *Science* **286**:2169–2172.

Hahne M, Zorn-Kruppa M, Guzman G, Brandner JM, Haltner-Ukomado E, Wätzig H, and Reichl S (2012) Prevalidation of a human cornea construct as an alternative to animal corneas for in vitro drug absorption studies. *J Pharm Sci* **101**:2976–2988.

Pescina S, Govoni P, Potenza A, Padula C, Santi P, and Nicoli S (2015) Development of a convenient ex vivo model for the study of the transcorneal permeation of drugs: histological and permeability evaluation. *J Pharm Sci* **104**:63–71.

Prausnitz MR and Noonan JS (1998) Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye. *J Pharm Sci* **87**:1479–1488.

Reichl S and Becker U (2008) Cell culture models of the corneal epithelium and reconstructed cornea equivalents for in vitro drug absorption studies, *in Drug Absorption Studies* (Ehrhardt C and Kim KJ eds) pp 283–306, Springer Science+Business Media, LLC, New York, NY.

Reichl S, Bednarz J, and Müller-Goymann CC (2004) Human corneal equivalent as cell culture model for in vitro drug permeation studies. *Br J Ophthalmol* **88**:560–565.

Rönkkö S, Vellonen KS, Järvinen K, Toropainen E, and Urtti A (2016) Human corneal cell culture models for drug toxicity studies. *Drug Deliv Transl Res* **6**:660–675.

Sasaki H, Yamamura K, Tei C, Nishida K, and Nakamura J (1995) Ocular permeability of FITC-dextran with absorption promoter for ocular delivery of peptide drug. *J Drug Target* **3**:129–135.

Scott L, Eskes C, Hoffmann S, Adriaens E, Alepée N, Bufo M, Clothier R, Facchini D, Faller C, Guest R, et al. (2010) A proposed eye irritation testing strategy to reduce and replace in vivo studies using bottom-up and top-down approaches. *Toxicol In Vitro* **24**:1–9.

Takezawa T, Aoki S, Oshikata A, Okamoto C, Yamaguchi H, Narisawa Y, and Toda S (2012) A novel material of high density collagen fibrils: a collagen xerogel membrane and its application to transplantation in vivo and a culture chamber in vitro, in *International Proceedings Division, ed.* 24th European Conference on Biomaterials; 2011 September 4–8; Dublin, Ireland. pp. 181–185, Medimond International Proceedings, Bologna, Italy.

Takezawa T, McIntosh-Ambrose W, and Elisseeff JH (2008) A novel culture model of rabbit corneal epithelium utilizing a handy scaffold of collagen vitrigel membrane and its cryopreservation. *AATEX* **13** (Suppl):176.

Takezawa T, Nishikawa K, and Wang PC (2011) Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and the changes of its barrier function induced by exposing eye irritant chemicals. *Toxicol In Vitro* **25**:1237–1241.

Takezawa T, Nitani A, Shimo-Oka T, and Takayama Y (2007a) A protein-permeable scaffold of a collagen vitrigel membrane useful for reconstructing crosstalk models between two different cell types. *Cells Tissues Organs* **185**:237–241.

Takezawa T, Ozaki K, Nitani A, Takabayashi C, and Shimo-Oka T (2004) Collagen vitrigel: a novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. *Cell Transplant* **13**:463–473.

Takezawa T, Ozaki K, and Takabayashi C (2007b) Reconstruction of a hard connective tissue utilizing a pressed silk sheet and type-I collagen as the scaffold for fibroblasts. *Tissue Eng* **13**:1357–1366.

Takezawa T, Takeuchi T, Nitani A, Takayama Y, Kino-Oka M, Taya M, and Enosawa S (2007c) Collagen vitrigel membrane useful for paracrine assays in vitro and drug delivery systems in vivo. *J Biotechnol* **131**:76–83.

Xu YG, Xu YS, Huang C, Feng Y, Li Y, and Wang W (2008) Development of a rabbit corneal equivalent using an acellular corneal matrix of a porcine substrate. *Mol Vis* **14**:2180–2189.

Yamaguchi H, Kojima H, and Takezawa T (2013) Vitrigel-eye irritancy test method using HCE-T cells. *Toxicol Sci* **135**:347–355.

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