Air-Interface Condition Promotes the Formation of Tight Corneal Epithelial Cell Layers for Drug Transport Studies

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Purpose. To identify the growth conditions that would favor the development of a functional primary culture of pigmented rabbit corneal epithelial cells on a permeable support comparable to the intact tissue in bioelectric properties.

Methods. Rabbit corneal epithelial cells were isolated and cultured on precoated fibronectin/collagen/laminin permeable filters. Cells were grown at an air-interface with supplemented DMEM/F12 medium. Immunofluorescence and electron microscopy techniques, respectively, were used to confirm cornea-specific marker and morphological features. Permeability of the cell layers to model polar compounds was evaluated using ^{14}C-mannitol, fluorescein isothiocyanate (FITC) and fluorescein isothiocyanate-dextran of 4,000 molecular weight (FD4).

Results. We found that culturing the epithelial cells at an air-interface (AIC) was a critical factor in the formation of tight cell layer and that omitting fetal bovine serum and keeping the concentration of epidermal growth factor at 1 ng/ml were equally important. Phenotypically, the AIC cell layers were found to express cornea-specific 64 kD keratin. Compared with cells cultured under the liquid-covered (LCC) condition, those cultured under AIC exhibited a significantly higher peak transepithelial electrical resistance (TEER) of up to 5 kΩ.cm^2, a higher potential difference (PD) of up to 26 mV, and an estimated short-circuit current (I_{eq}) of 5 μA/cm^2 after 7–8 days of culture. These values were comparable to those in the excised cornea. Consistent with the TEER, the AIC cell layers were 4–40 times less permeable to paracellular markers than their LCC counterpart.

Conclusions. The AIC model merits further characterization of drug transport mechanisms as well as drug, formulation, physiological, and pathological factors influencing corneal epithelial drug transport.

KEY WORDS: cultured corneal epithelial cells; air-interface; paracellular permeability.

INTRODUCTION

Corneal drug transport studies are typically performed in isolated tissues mounted in modified Ussing chambers (1). Probably due to the technical difficulties with isolating and maintaining an intact epithelial sheet, the role of the corneal epithelia in overall drug transport is rarely studied directly, if at all. Rather, this is inferred, by comparing drug flux in the tissue stripped of its epithelial layers against that afforded by the intact tissue (1).

Primary cultured corneal epithelial cell layers grown on a permeable support promise to allow the direct resolution of questions concerning drug transport mechanisms as well as drug- and formulation factors influencing corneal epithelial drug transport. The advantages of such a model system include: (a) high sample throughput; (b) ability to access both the apical and basolateral sides directly in mechanistic transport studies; and (c) freedom from possible confounding interference of non-epithelial cells.

The idea of culturing corneal epithelial cells dated back to the 1950's (2). The goal then was to identify the cellular nutritional requirements for the preservation of corneal tissues. Since then, corneal epithelial cells have been cultured from rabbit, human, cow, monkey, rat and mouse, with rabbit being the most popular. These cultured cells were used to evaluate intrinsic and extrinsic cell differentiation and regulation (3,4), as well as cell attachment to extracellular matrix proteins (5). Typically, these corneal epithelial cells were cultured on plastic support submerged in growth medium containing fetal bovine serum (2–20%), fibroblast feeder layers, or growth supplements such as epidermal growth factor (EGF) (10 ng/ml), insulin (5 μg/ml) and cholera toxin (0.1 μg/ml) (6). Aside from the fact that plastic petri dish, by their impermeable nature, is not suitable for transepithelial drug transport studies, epithelial cells grown on a plastic support may not fully express their *in vivo* ion transport function that may be integral to secondary active drug transport (7).

Corneal epithelial cell layers cultured on a permeable support have been attempted on both cell lines (8) and primary cells (9). The SIRC cell line culture has been morphologically evaluated and was found to achieve an average maximum cell layer growth of 4.54 cell layers at day 10 (10). This cell line has also been used for cytotoxicity screening (10), assessment of cell life span (8), evaluation of basic plasma membrane transport processes (11) and testing of antiviral drug efficacy (12). Despite the claim of epithelial origin, the SIRC cells exhibit a fibroblast phenotype (13). Kawazu *et al.* (9), on the other hand, used preserved rabbit corneal epithelial cells. The resulting primary cell culture cell layers were, however, extremely leaky, as indicated by a TEER of 144 Ω.cm^2. This is far below the value of 3,000–8,000 Ω.cm^2 (14,15) for the excised rabbit corneal tissue.

Culturing cells under the air-interface condition (AIC) has been attempted for conjunctiva (16), airway (17) and epidermis (18). In the case of airway epithelial cells, culturing under the AIC condition resulted in enhanced morphological features, polarity, and ion transport characteristics, comparable to those of the native tissue (19). This was attributed to improved oxidative metabolism made possible by improved access of oxygen to the epithelial cells when cultured in the air-interface configuration (20). Thus, the purpose of our present study was to identify the growth conditions that would yield a functional primary culture of pigmented rabbit corneal epithelial cells that exhibits similar morphological characteristics, bioelectric parameters, and paracellular permeability to those observed in

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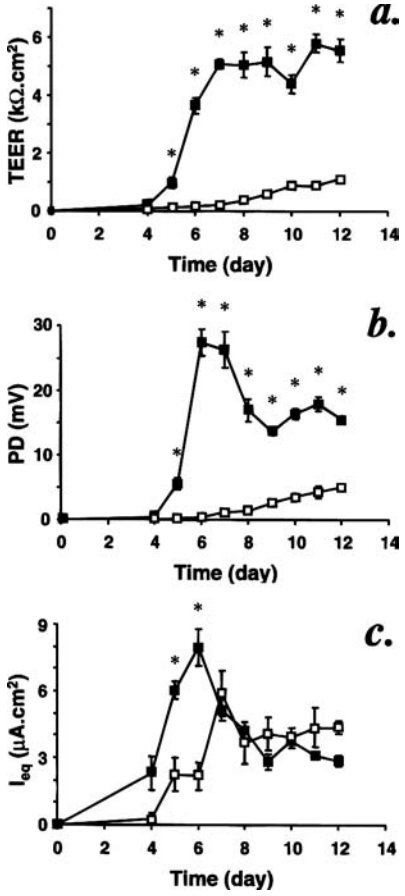

Fig. 1. Time course of bioelectric parameters in air-interfaced (■, AIC) and liquid-covered (□, LCC) rabbit corneal epithelial multilayers. Peak TEER, PD, and I_{eq} for AIC are 5.08 ± 0.17 kΩ.cm^2, 26.3 ± 2.8 mV, and 5.1 ± 1.0 μA · cm^2 (n = 10), respectively. The corresponding values for LCC are 0.21 ± 0.01 kΩ.cm^2, 1.1 ± 0.15 mV, and 5.9 ± 1.0 μA.cm^2 (n = 29). Error bars represent s.e.m. Asterisk denotes significant difference ($p < 0.001$) between AIC and LCC on a given day by Student's t-test. Where not shown, the error bar is smaller than the size of the symbol.

the intact tissue. We found that culturing the epithelial cells at an air-interface was a critical factor.

MATERIALS AND METHODS

Materials

Male Dutch-belted pigmented rabbits, weighing 2.5 kg, were purchased from Irish Farms (Los Angeles, CA). The investigations utilizing rabbits described in this report conformed to the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). Protease, epidermal growth factor (EGF), hydrocortisone, and fluorescein isothiocyanate-labeled dextran (FD-4) were purchased from Sigma Chemical (St. Louis, MO). Sodium fluorescein and fluorescein isothiocyanate (FITC) were from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). S-MEM, fetal bovine serum (FBS), gentamicin, Fungizone™ and DMEM/F12 media were from GIBCO BRL/Life Technologies (Gaithersburg, MD), while PC-1, penicillin, and streptomycin were from BioWittaker, Inc. (Walkersville, MD). Insulin-transferrin-selenium (ITS$^+$) premix, collagen, fibronectin, laminin, and bovine pituitary extract (BPE) were from Collaborative Biomedical (Bedford, MA). [^{14}C]-Mannitol was from Moravek Biochemicals, Inc. (Brea, CA).

Primary Culture of Rabbit Corneal Epithelial Cells

Rabbits were sacrificed by a rapid intravenous overdose of sodium pentobarbital (120 mg/kg body weight). Upon excision, the whole eyeball was placed in an eyeball holder that only exposed the epithelial layer to 0.2% protease in S-MEM for 60 min at 37°C. The suspension of dissociated epithelial cells was centrifuged at 200×g for 10 min. The resulting pellet was suspended in S-MEM containing 10% FBS, filtered through a 40 μm cell strainer, centrifuged and the pellet was resuspended in PC-1 or DMEM/F12 media supplemented with antibiotics (1 μg/ml Fungizone™ (amphotericin B), 50 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin), Insulin-transferrin-selenium (ITS$^+$) premix, 30 μg/ml bovine pituitary extract (BPE), 1 ng/ml EGF and 0.36 μg/ml hydrocortisone. Cell count and viability were assessed by 0.1% trypan blue exclusion. Unless otherwise indicated, cells were plated at 0.9×10^6 cells/cm^2 onto Costar 6.5-mm Clear Transwells pretreated with 3:1:1 proportion of collagen (3 mg/ml)/ fibronectin (1 mg/ml)/ laminin (1 mg/ml). Air-interface condition was created by removing media from the apical compartment and keeping 0.3 ml of media in the basolateral compartment on day 1. Cells were kept in a humidified incubator at 37°C in 95% air/5% CO_2, and media was changed every day. Transepithelial electrical resistance (TEER) and potential difference (PD) were measured with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) and corrected for the background contributed by the filter coated with only the extracellular matrix materials. I_{eq} (A/cm^2) was estimated from the ratio of PD over TEER.

Transmission Electron Microscopy

Primary cultured cells on day 7–8 were fixed in half-strength Karnovsky's 2% paraformaldehyde-2.5% glutaraldehyde fixative in phosphate buffer, postfixed in 1% osmium tetroxide (OsO_4), and stained with 1% uranyl acetate, each for 1 hr at 4°C before being dehydrated in a graded series of ethanol. For transmission electron microscopy, cultures were infiltrated and embedded in Epon, thin-sectioned (60–70 nm), placed on Formvar-coated slot grids, and stained with uranyl acetate and lead citrate for examination in a Hitachi H-7000 electron microscope.

Immunohistochemical Staining for Cornea-Specific Keratin

Primary cells cultured to day 7–8 were fixed with 4% paraformaldehyde/phosphate buffer saline, pH 7.4 (PBS) buffer for 1 hr and incubated overnight at 4°C in 30% sucrose/PBS (pH 7.4) solution. Cell layers were embedded in OCT compound (Miles, Elkhart, IN) and quick-frozen in liquid nitrogen. Cryostat sections of 8 μm thick were cut on a Tissue-Tek cryostat from each frozen specimen, placed on gelatin-coated slides, and

Table 1. Effect of Culture Media Composition on the Transepithelial Electrical Resistance (TEER), Potential Difference (PD), and Short-Circuit Current (I_{eq}) of Corneal Epithelial Cell Layers on Day 7–8 of Culture

Media Composition	TEER ($k\Omega.cm^2$)	PD (mV)	I_{eq} ($\mu A/cm^2$)
DMEM/F12 non-supplemented	0.054 ± 0.02*	0.49 ± 0.12*	33 ± 9.9*
DMEM/F12 + 1% FBS	1.63 ± 0.21*	8.3 ± 0.7*	5.5 ± 0.3
DMEM/F12 + 0 ng/ml EGF	3.11 ± 0.20	10.1 ± 0.4*	3.3 ± 0.2*
DMEM/F12 + 1 ng/ml EGF	**3.21 ± 0.03**	**22.0 ± 2.0**	**6.8 ± 0.6**
DMEM/F12 + 5 ng/ml EGF	1.52 ± 0.13*	14.9 ± 1.3	10.1 ± 0.9
DMEM/F12 + 10 ng/ml EGF	1.18 ± 0.06*	11.9 ± 0.8	10.1 ± 0.4
PC-1	2.26 ± 0.10*	6.3 ± 0.5*	2.8 ± 0.2*

Note: Media composition for all cultures was supplemented (see Materials and Methods), unless otherwise indicated. Optimal conditions are indicated in bold. Values are mean ± s.e.m., n = 5–23.

* Statistical significance was tested by one way ANOVA and the Fisher's PLSD post hoc test. Statistical significance for media composition analysis was set at $p < 0.05$ when compared with results from the DMEM/F12 + 1 ng/ml EGF culture condition. For all media composition analysis, cells were cultured at a plating density of 1.5×10^6 cells/cm^2.

Table 2. Effect of Seeding Density on the Transepithelial Electrical Resistance (TEER), Potential Difference (PD), and Short-Circuit Current (I_{eq}) of Corneal Epithelial Cell Layers on Day 7–8 of Culture

Cells/cm^2	TEER ($k\Omega.cm^2$)	PD (mV)	I_{eq} ($\mu A/cm^2$)
(A) 1.5×10^6	3.21 ± 0.03*	22.0 ± 2.0	6.9 ± 0.6
(B) 0.9×10^6	**5.08 ± 0.17**	**26.3 ± 2.8**	**5.1 ± 0.5**
(C) 0.6×10^6	3.40 ± 0.08*	20.4 ± 1.0	6.0 ± 0.3
(D) 0.3×10^6	2.34 ± 0.16*	19.7 ± 1.3	8.6 ± 0.8

Note: Media composition for all cultures was supplemented (see Materials and Methods), unless otherwise indicated. Optimal conditions are indicated in bold. Values are mean ± s.e.m., n = 5–23.

* Statistical significance was tested by one way ANOVA and the Fisher's PLSD post hoc test. In this analysis, cells were placed under AIC condition on day 1. The TEER showed statistical significance between groups A and B, A and D, B and C, B and D, C and D; PD and I_{eq} were not statistically significant ($p > 0.05$).

Table 3. Effect of Air-Interfaced Condition (AIC) on the Transepithelial Electrical Resistance (TEER), Potential Difference (PD), and Short-Circuit Current (I_{eq}) of Corneal Epithelial Cell Layers on Day 7–8 of Culture

Day	TEER ($K\Omega.cm^2$)	PD (mV)	I_{eq} ($\mu a/cm^2$)
1	**5.08 ± 0.17**	**26.3 ± 2.8**	**5.1 ± 0.50**
2	4.20 ± 0.23	10.8 ± 1.4*	2.5 ± 0.31*
3	3.61 ± 0.20*	7.7 ± 1.4*	2.0 ± 0.31*

Note: Media composition for all cultures was supplemented (see Materials and Methods), unless otherwise indicated. Optimal conditions are indicated in bold. Values are mean ± s.e.m., n = 5–23.

* Statistical significance was tested by one way ANOVA and the Fisher's PLSD post hoc test. For the effect of AIC condition, cells were seeded at an optimal seeding density of 0.9×10^6 cells/cm^2 as cells were placed under AIC condition at day 1, day 2, and day 3. The TEER was statistically significant between day 1 and day 3; PD and I_{eq} were statistically significant between day 1 and day 2 as well as between day 1 and day 3 ($p < 0.05$).

air dried overnight at 4°C. Non-specific binding was blocked by incubating the samples in 0.1 M cacodylate/1% BSA/0.1% Triton X-100 in PBS for 10 min at room temperature. The samples were incubated with the primary antibody (IgG), monoclonal mouse anti-epithelial keratin-AE5 (1:50 dilution) (ICN Pharmaceuticals, Inc., Costa Mesa, CA) for 30 min at 37°C, gently washed three times with PBS, and then incubated with the secondary antibody coupled rhodamine (CY3-rabbit anti-mouse IgG , Chemicon, Temecula, CA) at 1:100 dilution for 30 min at 37°C. Those samples not treated with the primary antibody served as the negative control.

Paracellular Permeability Assessment

All permeability experiments were performed in Bicarbonated Ringer's Solution (BRS) at an osmolality of 300 mOsm/kg under a humidified atmosphere of 5% CO_2/95% air at 37°C. Transport was initiated by dosing the apical compartment with BRS containing a paracellular marker. The paracellular markers were: (a) [^{14}C]-Mannitol (MW = 182.2; 5 μCi/ml (100 μM)); specific radioactivity, 50 mCi/mmol; (b) Na fluorescein (MW = 376; 0.2 mg/ml); (c) FITC, MW = 389; 0.25 mg/ml); and (d) FD-4 (MW = 4,400; 5 mg/ml). Fluorescein, FITC and FD-4 were dosed on the mucosal side. Samples of 200 μl were collected from the basolateral side every 30 or 60 minutes for up to 4 hours. Radiolabeled samples were mixed with liquid scintillation cocktail (Econo-safe, Research International, Mount Prospect, IL) for assay using a liquid scintillation counter (LS 1801, Beckman, Fullerton, CA) and the fluorinated samples were analyzed in a Hitachi fluorescence spectrophotometer. The 200 μl aliquot removed was immediately replenished with an equal volume of fresh buffer. The cumulative appearance of marker in the receiver compartment was plotted as a function of time. The steady-state flux (J) and the apparent permeability coefficient (P_{app}) were estimated by linear regression using the equation:

$$P_{app} = J/(AC_0) = (dQ/dt)/(AC_0) \quad (1)$$

where J = dQ/dt is the solute flux, A is the diffusional surface area (0.33 cm^2), and C_0 is the initial drug concentration.

Statistical Analysis

All data are presented as the mean ± standard error of the mean. Statistical significance was determined by Student's *t* test for unpaired sample assuming equal variance; $p < 0.05$ was considered significant. Statistical difference among multiple groups was determined by One Way Analysis of Variance (ANOVA) and group means were contrasted for significant differences using the Fisher's PLSD post hoc test; $p < 0.05$ was considered significant.

RESULTS

Bioelectric Parameters of Primary Cultured Rabbit Corneal Epithelial Cells

Our cell isolation method yielded 7–8 $\times$ 10^6 cells per animal, equivalent to 30 Transwell™ (0.33 cm^3) samples, with viability greater than 98%. Both AIC and LCC cell multilayers began to exhibit a measurable TEER and PD from day 3 onwards, reaching confluency on day 7–8. Compared with LCC, AIC developed a 5-fold higher TEER and a 3-fold higher PD (Fig. 1).

Of the two culture media we tested, cells grown in PC-1 media achieved a PD and a TEER value about two-thirds of that afforded by supplemented DMEM:F12 + 1 ng/ml EGF media, whereas DMEM:F12 non-supplemented resulted in ~60- and ~45-fold lower in TEER and PD, respectively (Table 1). Therefore, we used a supplemented DMEM:F12 medium in subsequent cultures. In addition, FBS was eliminated from the medium due to its adverse effect on the TEER and PD of the cell layers when compared to DMEM:F12 + 0 ng/ml EGF (Table 1). The EGF concentration was held at 1 ng/ml because cells grown in DMEM/F12 at 5 and 10 ng/ml concentrations resulted in a lower TEER (Table 1). Cells grown under no EGF resulted in significantly lower PD and I_{eq}. In terms of seeding density, 0.9 $\times$ 10^6 cells/cm^2 was found to be optimal (Table 2). At this seeding density, bioelectric parameters were found to be optimal when cells were placed under AIC condition on day 1 than on day 2 or day 3 (Table 3).

Morphological Assessment of AIC

The corneal epithelial cells gradually flattened as they approached the surface (Fig. 2b) and can be distinguished into basal, wing and flattened superficial cell layers. The superficial cells showed microvilli and the formation of tight junctions and desmosomes (Fig. 2a). Numerous fine cytoplasmic filaments were also present (Fig. 2c).

Immunohistochemical Staining of AIC

The cultured corneal epithelial cells stained positively for a cornea-specific basic K3 (64 kDa) keratin (21) with a commercially available monoclonal antibody (AE5) (Fig. 3b), as did the freshly excised tissue (Fig. 3a). The omission of primary antibody as negative control did not stain (Fig. 3c).

Paracellular Permeability Assessment of Corneal Epithelial Cell Multilayers

Table 4 shows that the paracellular permeability of AIC was comparable to that of the isolated tissue. Compared with

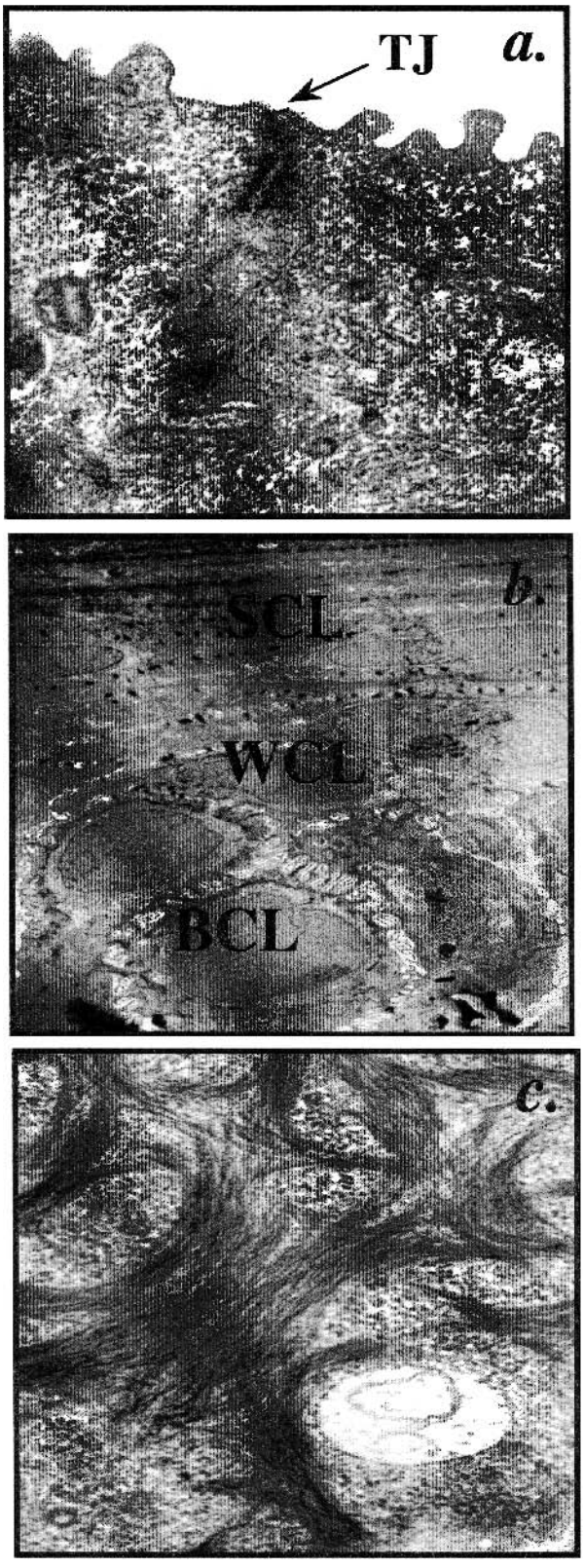

Fig. 2. Transmission electron micrograph of day 7 air-interface corneal epithelial cell culture illustrating: (a; ×21,800) tight junction (TJ), (b; ×8,000) the major cell layers of the corneal epithelium (SCL = superficial cell layers; WCL = wing cell layers; BCL = basal cell layer), and (c; ×47,700) numerous cytokeratin.

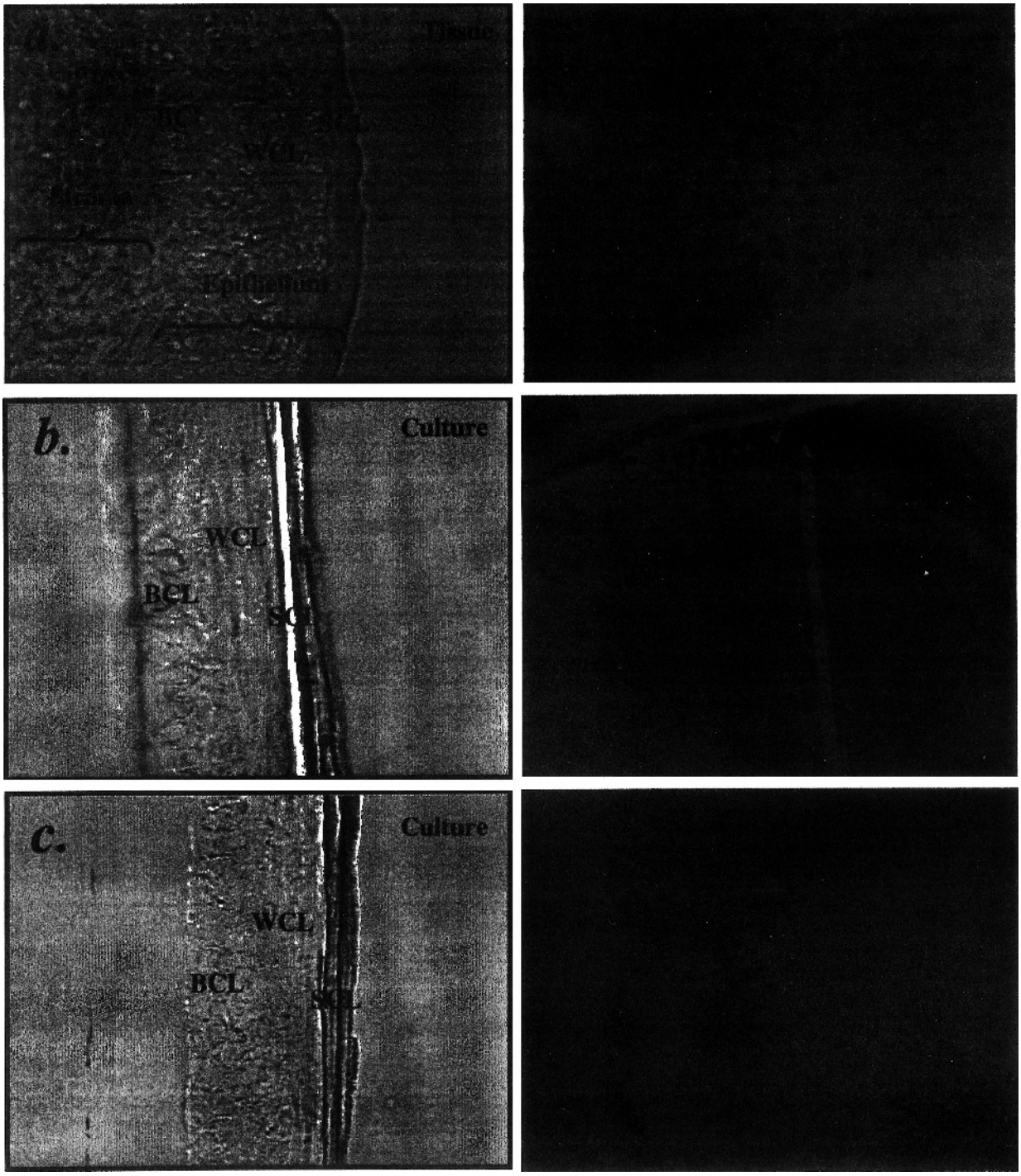

Fig. 3. Immunofluorescence staining for cornea-specific 64-kDa cytokeratin of AIC rabbit corneal epithelial cells: (*a*) positive staining of rabbit tissue corneal epithelium with AE5 monoclonal antibody (SCL = superficial cell layers; WCL = wing cell layers; BCL = basal cell layer), (*b*) positive staining of day 7 cultured corneal epithelial cells with AE5 and (*c*) negative control of day 7 cultured corneal epithelial cells. The corresponding phase-contrast micrographs are in the left-hand column.

the AIC, the LCC was 44 times more permeable to mannitol, 4 times to fluorescein, 5 times to FITC, and 37 times to FD-4.

DISCUSSION

An important finding in the present study is that implementing the air-interface condition on day 1 of seeding is critical to the formation of tight junctions, as indicated by TEER (Fig. 1) and the cell layers' permeability to paracellular markers (Table 2). On this basis alone, our primary corneal epithelial cell culture grown on a permeable support is superior to that reported by Kawazu *et al.* (9). Their culture model was characterized by a peak TEER of 144 $\Omega.cm^2$, 35 times lower than that afforded by our AIC model but comparable to that afforded

Table 4. Permeability Coefficient (P_{app}) of Paracellular Marker Solutes in Air-Interfaced and Liquid-Covered Cultured Rabbit Corneal Epithelial Cell Multilayers Compared with that in the Excised Cornea

Agent	MW	P_{app} (10^{-6} cm/s) AIC	P_{app} (10^{-6} cm/s) LCC	Tissue
[^{14}C]-Mannitol	182	0.044 ± 0.0036	1.93 ± 0.12	0.1[a]
Fluorescein	376	0.32 ± 0.01	1.13 ± 0.15	0.43 ± 0.09[b]
FITC	389	0.20 ± 0.017	1.07 ± 0.14	nd
FD-4	4,400	0.018 ± 0.003	0.66 ± 0.09	0.056 ± 0.016[c]

Values are mean ± s.e.m., n = 4–6; nd = not determined.
[a] From ref. (15).
[b] From ref. (11).
[c] From ref. (26).

by our LCC model. Therefore, it is not surprising that their drug permeability values varied from as low as 2-fold (e.g., propranolol) to as high as 37-fold (e.g., atenolol) higher than those in the excised tissue (22).

Key differences between Kawazu *et al.*'s model (9) and our AIC model include: (a) source of corneal epithelial cells, (b) condition of these cells before and during culture preparation, (c) seeding density, (d) substrata, (e) media supplements, and (f) air-interface condition. Specifically, whereas Kawazu *et al.* used corneal epithelial cells that were supplied frozen in 10% DMSO and 10% FBS in RCGM2 by Kurabo Industries Ltd. (Osaka, Japan), we used freshly isolated pigmented rabbit corneal epithelial cells. In terms of substrata, Kawazu *et al.* (9) precoated their collagen Transwell-COL with fibronectin, whereas we precoated ours with collagen, fibronectin and laminin. Although we did not carry out an extensive study on the effect of substrata, it is important to note that this factor may have played an important role in the overall result. In terms of seeding density, Kawazu *et al.* (9) used 4×10^4 cells/cm^2, with cell detachment occurring at higher values. We used a seeding density of 0.9×10^6 cells/cm^2 and did not observe cell detachment at any seeding density.

In terms of media composition, Kawazu *et al.* used DMEM/F12 supplemented with 5% FBS, 10 ng/ml EGF, 0.1 μg/ml cholera toxin, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. We also used DMEM/F12 but without supplementation with FBS and cholera toxin. We found that, supplementing the medium with 1 ng/ml EGF resulted in a 2-fold increase in PD and I_{eq}, when compared with no EGF (Table 1). At higher EGF concentrations (5 and 10 ng/ml), the TEER decreased by >2-fold, whereas the PD and I_{eq} remained unchanged. Serum was found to inhibit formation of tight junction (lower TEER) in AIC cultured corneal epithelial cells. This effect has also been seen in distal human fetal lung (23), choroid plexus (24), and retinal pigment epithelial cell cultures (25). Moreover, we used a 10 times lower EGF concentration and included in our media bovine pituitary extract (BPE), bovine serum albumin (BSA), L-glutamine, transferrin, selenious acid, linoleic acid, gentamicin, and amphotericin B. Compared with DMEM/F12 non-supplemented media, our supplemented DMEM/F12 + 1 ng/ml EGF prototype media yielded up to 60- and 45-fold higher TEER and PD, respectively (Table 1).

We evaluated the effect of culturing corneal epithelial cells under air-interface condition on day 1, 2, and 3. Placing these cells under air-interface condition on day 1 compared to day 3 was found to facilitate the formation of tight junctions (Table 3). The resulting cell layers demonstrated the expected inverse dependence of permeability to the m.w. of paracellular markers, as in the case of conjunctival epithelial cell layers (16). Moreover, the 5 times lower P_{app} for mannitol in the cultured corneal epithelial cell layers than that in cultured conjunctival epithelial cell layers is consistent with their 5 times larger TEER.

In summary, a functional primary culture of rabbit corneal epithelia cells on a permeable support under the AIC condition, that potentially may amplify sample throughput by a factor of 15, has been established. Studies are under way to characterize in detail their ion and drug transport characteristics. We feel that with suitable modification of the culture conditions we developed, it may be possible to culture human primary corneal epithelial cell layers on a permeable support.

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