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Research Report

Establishment of an in vitro brain barrier epithelial transport system for pharmacological and toxicological study

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Abstract

An immortalized Z310 murine choroidal epithelial cell line was recently established in this laboratory. The purposes of this study were (1) to investigate the presence of tight junction (TJ) proteins in Z310 cells and (2) to develop a Z310 cell-based in vitro brain barrier transport model. Real-time RT-PCR studies revealed that Z310 cells possess mRNAs encoding ZO-1, -2, and -3, claudin-1, -2, -4, and -8, occludin, and connexin-32. Confocal microscopic analyses confirmed the presence of claudin-1 and ZO-1 in Z310 cells at cell–cell contact sites. When Z310 cells were grown on a two-chamber Transwell device, the [^{14}C]sucrose permeability coefficient and transepithelial electrical resistance (TEER) across the cell monolayer were 6×10^{-4} cm/min and 61 Ω-cm^2, respectively. To improve the tightness of Z310 barrier, the cells were cultured in astrocyte-conditioned medium (ACM), or in the presence of eicosapentaenoic acids (EPA, 10 μM), epidermal growth factor (EGF, 100 ng/mL), or dexamethasone (1 μM) in the growth medium. Treatment with ACM, EPA, EGF and dexamethasone significantly increased the TEER by 33%, 38%, 40%, and 50% above controls, respectively. However, only dexamethasone significantly reduced [^{14}C]sucrose paracellular permeability (−231% of controls). These data suggest that Z310 cells possess the TJ proteins. The presence of dexamethasone in the growth medium improves the tightness of Z310 cell monolayer to the level better than that of the primary culture of choroidal epithelial cells. The Z310 cell-based in vitro model appears to be suitable for transepithelial transport study of drugs and toxicants.

Theme: Cellular and molecular biology
Topic: Blood–brain barrier

Keywords: Choroid plexus; Z310; Blood–CSF barrier; Brain barrier; In vitro model; Dexamethasone

1. Introduction

The brain barrier that separates the systemic circulation from the cerebrospinal fluid (CSF) compartment is known as the blood–CSF barrier (BCB), primarily located in the choroid plexus. The BCB functions to restrict the entrance of substances from the blood to the CSF, actively secrete the CSF into brain, produce and secrete critical molecules such as transthyretin, vasopressin and transferrin, into the brain [5,44]. Since the endothelial cells in the choroid plexus are not at all tight but leaky, the passage of substances across the BCB is controlled by the apical layer of epithelial cells, which are tightly connected with each other through the tight junctions (TJ). Thus, the structural basis of the BCB is the TJs between choroidal epithelia.

The TJs in the BCB is a combination of transmembrane and cytoplasmic proteins linked to an actin-based cytoskeleton that allows the TJ to form a seal while being capable of rapid modulation and regulation. The intramembrane strands are formed by non-covalently linked branched polymers containing claudins and occludin, two major tetra-transmembrane components of TJs [35]. Occludin is an integral membrane protein, whereas the claudin family

Abbreviations: ACM, astrocyte-conditioned medium; CP, choroid plexus; EGF, epidermal growth factor; EPA, eicosapentaenoic acids; TJ, tight junction; TEER, transepithelial electrical resistance; SFM, serum free medium

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provides the primary seal to the TJs [11,13,35]. The cytoplasmic proteins interacting with the transmembrane strands include zonula occludens proteins (ZO-1, ZO-2, and ZO-3), AF6, 7H6 antigen, and cingulin [12,39]. A primary function of these cytoplasmic proteins is to form a protein network linking the junctional membrane components to the cytoskeleton proteins. One of the major cytoskeletal proteins is actin with known binding sites for all ZO proteins, claudins as well as occludin [20].

Increasing research effort in drug discovery, toxicant transport, and roles of BCB in neurological diseases demands establishment of an in vitro BCB model to suffice the research needs in academia and industry. In vitro BCB transport model using primary culture grown on Transwell devices has been established [27,49]. However, the impurity of cell population, the limited capacity of cell differentiation and passage, and the high animal costs have hampered the application of primary choroidal epithelial cells in basic research. To circumvent these limitations, an immortalized rat choroidal epithelial cell line, namely Z310 cells, has recently been created in this laboratory [47]. The cell line possesses the essential morphology of the parent primary cells, expresses the marker of transthyretin (TTR), and forms a confluent monolayer upon growing on semipermeable membrane, which restricts a paracellular leakage marker, [^{14}C]sucrose. However, the tightness as measured by trans-epithelial electrical resistance (TEER) remained to be improved in comparison to the model based on primary culture of choroidal epithelia.

Several methods have been suggested capable of improving the tightness of in vitro barrier. For example, Change et al. [4] have reported that withdrawal of serum from the growth culture medium drastically improve the barrier properties. Stevens et al. [32] have shown that human intestinal epithelial T-84 cancer cell line can respond to the stimulation by interleukin-15 (IL-15), resulting in an increased TEER. Janzer and Raff [21] have demonstrated that incubation with astrocyte conditioned medium induces brain barrier characteristics. A study by Yamagata et al. [42] has revealed that eicosapentaenoic acids (EPA) and gamma linolenic acid (GLA) increase the trans-endothelial electrical resistance in porcine brain capillary endothelial monolayer. More recently, Singh and Harris [30] have proved that epidermal growth factor (EGF) induces a 3-fold increase in TEER values using MDCK type II cells. Furthermore, dexamethasone, a synthetic glucocorticoid, has been reported to improve TEER and to reduce the paracellular permeability in cultured 31EG4 mouse epithelial cells [43].

The purposes of this study were to identify and characterize the tight junction proteins in Z310 cells by using confocal microscopy and real-time RT-PCR techniques, and to establish a Z310 cell based in vitro BCB transport model. For the latter aim, we have used the following four approaches to improve the tightness of Z310 barrier: (1) by using serum free medium (SFM) and astrocyte-conditioned medium (ACM), (2) by treating Z310 cells with dexamethasone or IL-15 to increase recruitment of TJ proteins to cell–cell contact site, (3) by introducing IL-15 or polyunsaturated fatty acids (EPA, GLA) into the culture medium to stimulate the production of TJ proteins, and (4) by improving the cell differentiation with EGF. Both TEER, an instantaneous measurement of ionic conductivity, and [^{14}C]sucrose paracellular leakage, a paracellular slow diffusion marker, were used to evaluate the tightness of Z310-derived BCB model in comparison to HT22 cell-based monolayer as the negative control and MDCK cell-based monolayer as the positive control. We used the barrier model derived from primary rat choroidal epithelial cells as the bench marker.

2. Material and methods

2.1. Materials

Chemicals and assay kits were obtained from the following sources: Dulbecco's modified essential medium (DMEM), Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), penicillin and streptomycin, gentamycin from Gibco (Grand Island, NY); antibiotic solution (100×), cis-hydroxyproline, L-glutamine, eicosapentaenoic acids (EPA), gamma linolenic acid (GLA), poly-L-lysine, epidermal growth factor (EGF), hydrocortisone, laminin, collagen solution (type I), and alanine from Sigma (St. Louis, MO); interleukin-15 (IL-15) and dexamethasone from Calbiochem (San Diego, CA); [^{14}C]Sucrose (specific activity: 55 mCi/mmol) from Moravek Biochemicals (Brea, CA); RNeasy mini kit and RNase-free DNase set from Qiagen (Valencia, CA); $MgCl_2$ solution, PCR buffer, dNTP, Oligo dT and MuLV reverse transcriptase from Applied biosystems (Foster city, CA); absolute QPCR Sybr Green Mix from ABgene (Rochester, New York); and DEPC water and Trizol reagent from Invitrogen (Carlsbad, CA). MDCK cell line and Eagle's Minimum Essential Media (EMEM) were purchased from American Type Culture Collection (ATCC). HT22 cell was a courtesy gift from Dr. Isom of Purdue University. Transwell-COL culture wells were purchased from Costar (Cambridge, MA).

2.2. Culture of Z310, MDCK, and HT22 cell lines

Detailed procedure to culture Z310 cells has been described in our previous publication [47] and followed in this study. Madin–Darby canine kidney (MDCK) cell line, originally developed from canine renal collecting duct epithelia, possesses the TJ property, and was used as the positive control. HT22, a mouse hippocampal cell line, can also form the monolayer, yet lacking the tight junction property. The HT22 cells were used as a negative control. Both Z310 and HT22 cells were maintained in DMEM (high glucose) medium supplemented with 10% FBS, 100

U/mL penicillin, 100 μg/mL streptomycin, and 40 μg/mL of gentamycin in a humidified incubator with 95% air–5% CO_2 at 37 °C. Z310 cells were passaged twice a week. MDCK cells were cultured with EMEM medium supplemented with 10% FBS.

2.3. Primary culture of rat choroid plexus epithelial cells

Primary culture of rat choroidal epithelial cells was established as a benchmark for this study. Choroidal epithelial cells were cultured using the method established in this laboratory [48]. The protocols that involve use of animals for primary culture have been approved by Purdue Animal Care and Use Committee at Purdue University. The plexuses from Sprague–Dawley rats (4–6 weeks old) (Harlan, Indianapolis, IN) were dissected and digested in HBSS containing 0.2% pronase at 37 °C for 5 min. The dissociated cells were washed in DMEM with 100 U/mL each of penicillin and streptomycin, and re-suspended in normal growth medium of DMEM supplemented with 10% FBS and 10 ng/mL EGF. The cells were plated in 35-mm Petri dishes at the density of $0.5–1 \times 10^6$ cells/mL and cultured in a humidified incubator with 95% air–5% CO_2 at 37 °C. The growth medium was replaced 3 days after the initial seeding and every 2 days thereafter. The culture showed a dominant polygonal type of epithelial cells for at least 7–10 days. At confluence, they were transferred to a two-chamber Transwell-COL wells for TEER measurement and [^{14}C]sucrose permeability study.

2.4. Primary culture of rat brain astrocytes

In order to obtain astrocyte-conditioned medium, a primary culture of astrocytes was established according to the procedure described by Goldman et al. [14] and used previously in this laboratory [45]. In brief, new-born rat brains were removed from skull and placed in a culture dish. The meninges and blood vessels on the surface were removed. Cerebellum was cut off and the rest brain was dropped in a beaker containing 5 mL sterile HBSS solution, minced with a fine ophthalmologic scissors about 5 min while swirling and digested in sterile HBSS containing 0.04% trypsin at 37 °C for 15 min. Digestion was stopped by adding trypsin inhibitor (0.05 mg/mL) and DNase (0.04 mg/mL). The cell suspension was further mechanically disrupted by passing through a fire-polished Pasteur pipette 20 times and filtrated through a Falcon™ 70-μm sieve. After centrifugation at 1000 × rpm for 5 min, the pellet was re-suspended in 20 mL of growth medium (DMEM + 10% FBS + antibiotics). The cells were counted and plated in T-75 culture flasks at the density of 1.8×10^6 cells/ flask in 15 mL of medium. They were cultured in a humidified incubator with 95% air–5% CO_2 at 37 °C. On the 7th day in culture, the tightly closed flasks were rotated at 200 rpm at 37 °C overnight on an orbital shaker. The medium was then replaced with 15 mL of fresh medium. Every 3 days thereafter, the medium was collected as astrocyte conditioned medium (ACM).

2.5. Preparation of rat brain capillary endothelial cells

Endothelial cells were isolated from rat brain capillaries as described previously [7]. In brief, the meninges of rat brain were removed and the cerebra were transferred to a pre-chilled glass homogenizer. The cerebra were mechanically homogenized in 3 volumes (based on tissue weight) of ice-cold homogenization buffer (2.603 g/L HEPES, 8.240 g/L NaCl, 0.298 g/L KCl, 0.174 g/L $MgSO_4 \cdot 7H_2O$, 0.138 g/L $NaH_2PO_4 \cdot H_2O$ and 0.399 g/L $CaCl_2 \cdot 2H_2O$) for 8 up-and-down strokes. A 4 volume of ice-cold 30% dextran-70 solution was added, followed by three additional strokes. The homogenate was centrifuged at 5400 × *g*, 4 °C for 15 min. Supernatant was carefully separated from the vasculature-enriched pellet, which was verified by light microscope examination. After 2–3 washes with the mixture of homogenization buffer and 30% dextran–70 solution (3:4), the pellet was used for total RNA extraction.

2.6. Quantitative real-time RT-PCR analysis of tight junction protein mRNAs

The mRNA expression levels of tight junction proteins were quantified using real-time RT-PCR analysis as described by Walker [36]. Briefly, total RNA was isolated from Z310 cells, rat brain choroid plexus and rat brain capillary using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by purification on RNeasy columns (Qiagen, Palo Alto, CA). Purified 1 μg of RNA was reverse transcribed with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and oligo-dT primers. The forward and reverse primers for target genes were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The ABsolute QPCR SYBR green Mix kit (ABgene, Rochester, New York) was used for real-time PCR analyses. The amplification was carried out in the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). Amplification conditions were 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C. All the primers for real-time PCR analyses were obtained from Sigma (St. Louis, MO) and the sequences are listed in Table 1.

2.7. Confocal immunofluorescence microscopy studies of ZO-1 and claudin-1

The distributions of typical tight junction proteins (Claudin-1 and ZO-1) in Z310 cell were studied using confocal microscopy. Z310 cells were plated on the Lab-Tek chambered slides (Lab-Tek, Rochester, NY) and grown until confluence (2–3 days). The slides were rinsed with PBS for three times. Cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min at 37 °C. Following

Table 1
Primer sequences for real-time PCR analysis of mRNAs encoding tight and gap junctional proteins

Name	Accession Number	Forward primer	Reverse primer	Product (bp)
Occludin	NM_031329	gggaatgtccagaacgagaaga	cgtggcaatgaacaccatga	82
Claudin-1	NM_031699	tcccaagccaacaccttctagt	gtattcgctccaggaggatctct	76
Claudin-2	XM_236535	tctgtggtgggcatgagatg	ctccacccactacagccactct	76
Claudin-4	XM_222088	tggagcctctgactagctttgac	gcttgcttagggagcgttagc	85
Claudin-8	XM_221709	catcaccggcttggttgtg	cagcgggttgtagaagtctctga	76
ZO-1	XM_218747	gcgaggcatcgttcctaataag	tcgccacctgctgtctttg	81
ZO-2	U75916	tcacagctgcaggtgtcaca	tgctgttctgggttgaggaact	80
ZO-3	XM_234924	gtaggtgaccctgcatctgtga	tccctgggtgggatgaca	77
Connexin 32	AH003192	agacacgcctgcatacattcc	ccaccctcagcttccaaactc	90
GAPDH	NM_017008	cctggagaaacctgccaagtat	agcccaggatgccctttagt	83
Beta-actin	V01217	tcctcctgagcgcaagtactct	gctcagtaacagtccgcctagaa	153

The sequences of primers were designed by using Primer Express 2.0 (Applied Biosystems, Foster City, CA).

incubation with 5% FBS in PBS for 10 min to block non-specific binding, the cells were incubated with primary antibodies (rabbit anti-ZO-1 or -claudin-1, Zymed, South San Francisco, CA) at room temperature for 4 h and then Flour Alexa-488 conjugated secondary antibody (goat anti-rabbit IgG, Molecular Probes, Eugene, OR) at 4 °C overnight. A small drop of mounting media was added to each well of the Lab-Tek and was covered with a coverslip. The slides were examined using confocal microcopy.

2.8. Culture in Transwell transport device

The Transwells with an insert diameter of 12 mm were used in this project. The permeable membranes divide the Transwell device into two compartments: the inner and outer chamber. Aliquots (0.9 mL) of cell suspensions (Z310, HT22, MDCK, or primary choroidal epithelial cells) were added to the inner chamber, which was inserted into the outer chamber containing 1.3 mL of the culture medium. The culture continued for 48 h and the medium was changed every 2 days thereafter.

The formation of confluent cell monolayers was judged by three criteria: (1) the cell grew to a confluent monolayer without visible spaces between cell clusters under a light microscope; (2) the height of the culture medium in the inner chamber had to be at least 2 mm higher than that in the outer chamber for at least 24 h; and (3) a constant TEER value across the cell layer was obtained. The TEER value was measured using an epithelial volt-ohmmeter (EVOM, World Precision Instruments, Sarasota, FL) after culturing in the Transwell chambers for at least 2 days. The net value was calculated by subtracting the background value, which was measured on collagen-coated, cell free chamber (blank), from the value of cell-seeded chamber.

The cultures that reached confluence, evidenced by the above-mentioned three criteria, were used for the [^{14}C]sucrose permeability study to investigate the paracellular diffusion across the cell monolayers. An aliquot (12.4 μL) of [^{14}C]sucrose stock solution (in 2% ethanol, specific activity: 55 mCi/mmol) was added into the outer chamber to a final concentration of 20 μM with 10.0 μCi [^{14}C]sucrose per mL of medium; subsequently, an aliquot (5 μL) of the medium was pipetted from the outer chamber and designated as time 0 sample. At each time point (3, 5, 15, and 30 min, 1 h, 2 h), an aliquot (20 μL) of medium was pipetted from the inner chamber (i.e., the receiver chamber) and replaced with an equal volume of fresh medium in order to keep the hydrostatic pressure. All the samples were mixed with Eco-lite cocktail and counted with a Packard Tri-Carb 2900 TR liquid scintillation analyzer (counting efficiency for ^{14}C: 95%).

To determine the permeability coefficients of [^{14}C]sucrose across the Z310 monolayer, the data within the linear range (<60 min) were used for linear regression analyses. The slope (mg/mL-min) of each data set was used to calculate the total and blank permeability coefficients in Eq. (1),

$$P_T \text{ or } P_B = \frac{V_R}{A \times C_D} \Delta C_R / \Delta t \quad (1)$$

where P_T represents the total permeability coefficient (cell monolayer + membrane + coating, cm/min); P_B, the blank permeability coefficient (membrane + coating, cm/min); V_R, volume of the receiver (0.9 mL); A, surface area of transport membrane (1.1 cm^2); C_D, sucrose concentration in donor chamber (mg/mL); C_R, sucrose concentration in receiver chamber (mg/mL). The permeability coefficient of epithelial barrier (P_E) is then obtained from the Eq. (2) [33,46],

$$\frac{1}{P_E} = \frac{1}{P_T} - \frac{1}{P_B} \quad (2)$$

where P_E is the permeability coefficient across cell monolayer.

2.9. Optimization of the initial culture condition for Z310 cell-based barrier model

The optimal growth of Z310 cells in the Transwell chamber depends upon the supporting materials used to construct the perforating membrane, the materials coated on the membrane, and the initial seeding cells. To optimize the growth condition for Z310 cells, we tested all these essential

factors. The filters attached to the inner chamber of Transwell device are made of three major types of membrane, i.e., polyester, polycarbonate, and collagen-coated polytetrafluoroethylene (PTFE). To screen the filter most suitable for Z310 cells, we pre-coated the filters with 0.01% collagen for 4–5 h, followed by UV irradiation overnight. Aliquots (0.9 mL) of Z310 cell suspension containing 2.0×10^5 cells were seeded onto each inner chamber and TEER was determined at confluence for comparison.

We further tested four types of coating materials, i.e., collagen, laminin, poly-lysine, and alanine in the Transwell inner chamber. Prior to the cell seeding, the inner chamber was treated with (1) 100 μL of 0.01% collagen for 4–5 h, followed by air dry overnight under UV light, (2) 100 μL of 14 μg/mL laminin for 10 min, followed by air dry for 45 min; (3) 100 μL of 0.01% poly-L-lysine for 5 min, followed by air dry overnight; or (4) 100 μL of 14 μg/mL alanine for 10 min, followed by air dry for 45 min. Aliquots (0.9 mL) of Z310 cell suspension containing 2.0×10^5 cells were seeded onto each well and TEER was determined at confluence.

The initial cell seeding numbers per inner chamber were tested by using Z310 cell suspension containing 5.0×10^4, 1.0×10^5, or 2.0×10^5 cells per well. The TEER values were determined at confluence.

2.10. Treatment to improve the tightness

Upon establishing the optimal growth condition for culturing Z310 cells in the Transwell chambers, Z310 cells were treated with modified media or chemicals to improve the tightness of the cellular barrier between two chambers. To test the ACM, aliquots (0.9 mL) of cell suspension containing 2.0×10^5 cells were seeded on collagen-coated polyester inner chambers. After 2 days in the culture, the medium was replaced with the ACM, which was collected from the primary culture of rat astrocytes, in both the inner and outer chambers, for the duration of the study. TEER and [^{14}C]sucrose permeability were determined as described above.

To study the effect of serum free media (SFM) on the barrier tightness, the cells were sequentially washed with PBS and SFM once after they grew to the confluence (4–6 days) in regular growth medium in the inner chamber. Two recipes of SFM were tested, one by Nitz et al. [26] by mixing DMEM with Ham's F12 medium at the ratio of 1:1, containing 0.7 mM L-glutamine, 100 μg/mL penicillin/streptomycin, 100 U/mL gentamicin, and 550 nM hydrocortisone, and the other by Zheng et al. [49] by supplementing DMEM with 5 μg/mL each of insulin and transferrin, 5 ng/mL each of sodium selenite and fibroblast growth factor, 10 ng/mL EGF, and 25 ng/mL PE1. The latter SFM was developed by this laboratory for primary culture of choroidal epithelial cells. The tightness of the cell monolayer was tested for the duration of the study.

To screen the effective reagents to increase the barrier tightness, the Z310 cells were harvested and re-suspended in normal culture medium supplemented with the following candidate reagents to the final concentration of (1) 10 μM EPA, (2) 10 μM GLA, (3) 100 ng/mL EGF, (4) 100 ng/mL IL-15, or (5) 1 μM dexamethasone. The cells were seeded to the inner chamber and the parameters of TEER and [^{14}C]sucrose permeability were determined for the duration of the study.

For each experiment, groups of the blank (with coating, but cell-free) and control (with coating and cells, but without treatments) were also included.

2.11. Statistic analysis

Statistical comparisons were performed using one-way ANOVA (Prism 3.0, GraphPad software Inc., San Diego, CA). A probability of $P < 0.05$ was considered statistically significant. Linear regression analyses were performed with KaleidaGraph (version 3.6, Synergy software, Reading, PA). The quality of fit was determined by evaluating the coefficient of determination (γ^2). Data were reported as mean ± SD.

3. Results

3.1. Presence of mRNAs encoding typical tight junctional proteins in Z310 cells

Total RNA fractions extracted from Z310 cells, rat brain capillary, and choroid plexus tissues were used to determine the presence of mRNAs encoding typical TJ proteins using real-time RT-PCR. The relative differences in mRNA expression of claudin-1, -2, -4, -8, ZO-1, -2, -3, occludin, and connexin 32 between cells and tissues were expressed using cycle numbers of threshold (Ct), which were normalized with that of GAPDH in the same sample. The percentages of mRNA expression in Z310 cells or brain capillary were calculated by referencing the mRNA levels in the choroid plexus tissue as 100%. Data presented in Table 2 showed that the mRNA levels of ZO-1, claudin-1, and claudin-2 were significantly lower in Z310 cells than those in rat choroid plexus tissue ($P < 0.001$). However, Z310 cells showed a higher expression of ZO-2 mRNA than did the choroid plexus ($P < 0.001$).

3.2. Distribution of ZO-1 and claudin-1 in Z310 cells

Confocal microscopic studies illustrated that Z310 cells had the positive staining for both ZO-1 and claudin-1, further confirming the presence of these TJ proteins in this cell line. Interestingly, the fluorescent signal by ZO-1 was primarily located, peripherally, on the cell-to-cell interface, suggesting a direct involvement of ZO-1 in the structure of TJ. By contrast, the distribution of claudin-1 signal in Z310 cells appeared to be rather diffusive, not only at the cell-to-cell interface, but also in cytoplasm (Fig. 1).

Table 2
Expression of mRNAs encoding tight junction and gap junction proteins in Z310 cells, rat brain capillary fraction, and rat choroid plexus

Target Genes	Rat choroid plexus	Z310 cells	Rat brain capillary
Occludin	100 ± 17%	69 ± 32%	230 ± 130%
ZO-1	100 ± 19.2%	4.2 ± 1.5%***	40.4 ± 33.8%***
ZO-2	100 ± 38.7%	1258 ± 171%***	483.9 ± 261.3%*
ZO-3	100 ± 16%	8.3 ± 3.7%**	191.4 ± 180%
Claudin-1	100 ± 35.6%	0.04 ± 0.008%***	1.96 ± 0.77%***
Claudin-2	100 ± 33.5%	5.0 ± 4.45%***	80 ± 1.95%*
Claudin-4	100 ± 47.6%	104.8 ± 114.3%	797.6 ± 1190.5%
Claudin-8	100 ± 59.1%	4.5 ± 3.6%	245.5 ± 195.5%
Connexin 32	100 ± 6.7%	19.1 ± 14.5%	909.1 ± 745.5%

Data represent mean ± SD, $n = 4–8$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3.3. Optimal culture conditions for Z310 cells in inner chambers

Z310 cells appeared to grow normally in all three tested filters. Upon reaching the confluence, the TEER values of Z310 monolayer on the polyester membrane were significantly higher than those grown on the polycarbonate and PTFE membrane ($P < 0.01$ and 0.001, respectively, Fig. 2A). Among tested coating materials, the TEER values of collagen coating were significantly higher than those with poly-L-lysine coating; however, no statistical significant differences were observed among coatings with collagen, laminin, and alanine (Fig. 2B). Neither did the initial seeding numbers (5.0×10^4, 1.0×10^5, and 2.0×10^5 cells/well) appear to affect the TEER values (Fig. 2C). Noticeably, however, the cells with initial low seeding numbers (5.0×10^4 or 1.0×10^5) took a longer time (4–6 days) to reach confluence than the cells with a higher initial seeding numbers (typically 2–3 days). Too high an initial cell seeding (e.g., 2.0×10^6), on the other hand, significantly

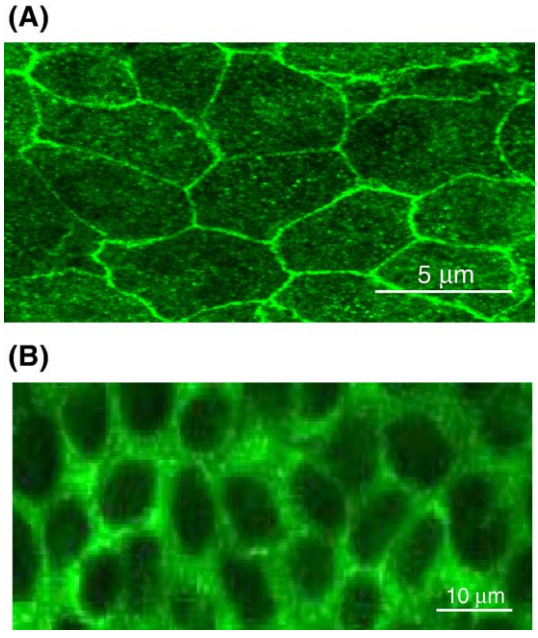

Fig. 1. Confocal microscopic study of subcellular distribution of selected tight junctional proteins in Z310 cell monolayer. Z310 were grown to confluence on the chambered Lab-Tek slide. After incubation with antibodies, the preparations were checked with a confocal microscope. (A) Peripheral distribution of ZO-1 on cell-to-cell contact membrane. (B) Diffusive distribution of claudin-1.

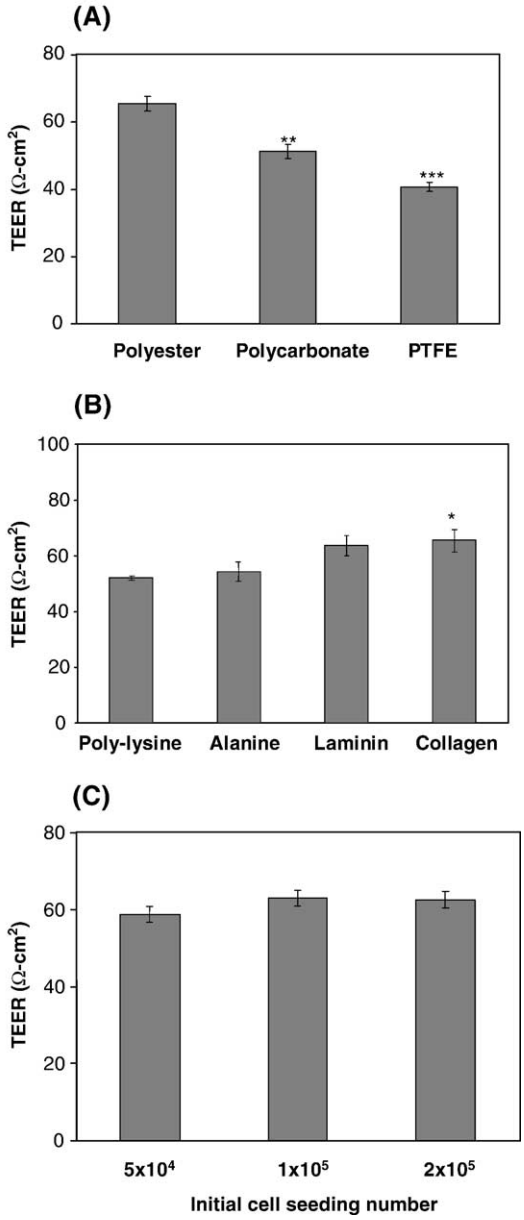

Fig. 2. Effect of filer types, coating materials, and initial seeding numbers on the TEER values. In all cases, the TEER values were determined at confluence. (A) All tested filters were pre-coated with 0.1% collagen for 4–5 h prior to cell seeding. The initial seeding numbers were 2.0×10^5 per well. (B) The polyester filter was pre-treated with various coating materials prior to cell seeding. The initial seeding numbers were 2.0×10^5 per well. (C) Cells were seeded onto polyester filters pre-coated with 0.1% collagen for 4–5 h. The initial seeding numbers were 5.0×10^4, 1.0×10^5, or 2.0×10^5 per well. Data represent mean ± SD, $n = 4–6$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

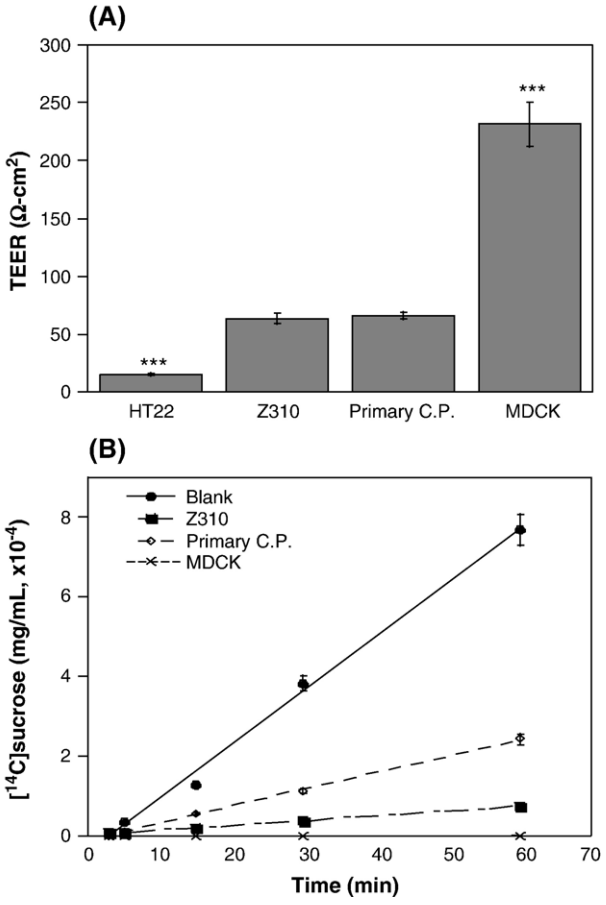

Fig. 3. Comparison of tightness of monolayers formed by Z310 cells, HT22 (as the negative control), MDCK (as the positive control), or primary choroidal epithelial cells (as bench marker control). (A) The TEER values were determined at confluence. Data represent mean ± SD, $n = 3–6$. $^{**}P < 0.01$ as compared to Z310 cells. (B) Paracellular leakage was determined by [^{14}C]sucrose permeability coefficients from 10-day cultures.

increased the gap spaces among the cell clusters by yet-undefined mechanism, possibly due to local over-confluence (data not shown).

3.4. Default values of TEER and [^{14}C]sucrose permeability coefficient

By using polyester filter and collagen coating with the initial seeding number of 2.0×10^5, we compared the TEER values and [^{14}C]sucrose paracellular permeability coefficients of Z310 cell-based barrier model with the control models based on monolayers of primary choroidal epithelia, HT22 cells (as a negative control), and MDCK cells (as a positive control). The TEER values in Z310 model (61.3 ± SD 2.62 Ω-cm^2) were significantly higher than those in HT22 cell monolayers (13 ± 0.95 Ω-cm^2), but significantly lower than those in MDCK cell monolayer (231.1 ± 11.0 Ω-cm^2), while it was not statistically significant in comparison to the monolayer formed from primary plexus cells (64.0 ± 1.66 Ω-cm^2) (Fig. 3A).

The paracellular permeability coefficients of [^{14}C]sucrose in Z310, primary plexus epithelia and MDCK were (5.98 ± SD 0.27) $\times 10^{-4}$ cm/min, (9.46 ± 0.45) $\times 10^{-4}$ cm/min, and (2.36 ± 0.22) $\times 10^{-5}$ cm/min, respectively. Since HT22 cells were detached from the membrane right after the formation of monolayer, [^{14}C]sucrose permeability study was not conducted on HT22 cells (Fig. 3B).

3.5. Improvement of tightness of Z310 barrier by dexamethasone

When Z310 cells in the inner chamber were incubated with the medium containing 1 μM dexamethasone, a glucocorticoid agonist, in both the inner and outer chambers, the TEER was increased by 10% above the control values at day 2, followed by a continued increase for up to 10 days after treatment (Fig. 4A). The maximal increase was observed at day 8, with the TEER of 106.5 ± 3.8 Ω-cm^2, which was about 50% of increase over the control, non-treatment group (61.3 ±

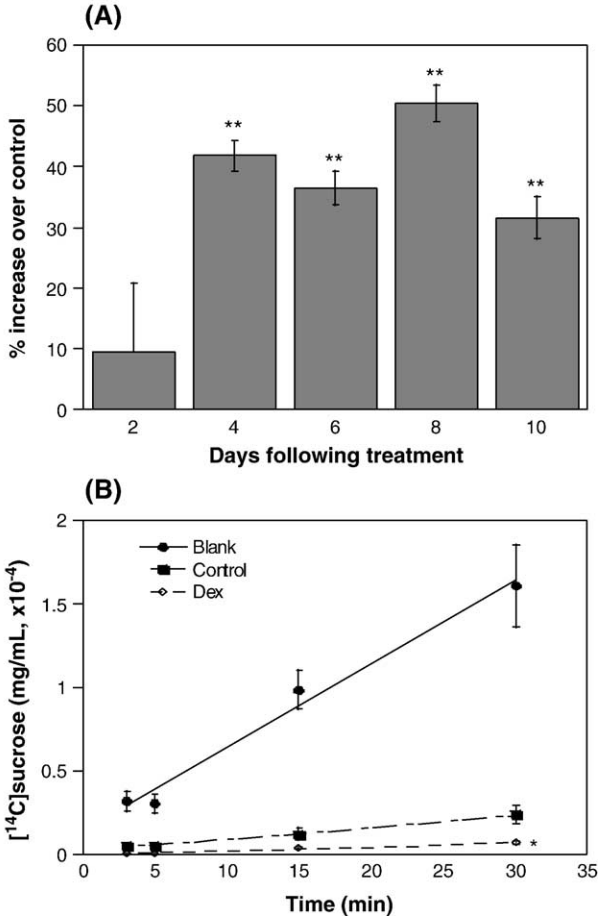

Fig. 4. Increase of tightness of the Z310 monolayer barrier by dexamethasone. Dexamethasone (1 μM) was added to the culture medium of both chambers upon cell seeding and maintained throughout the entire experiment. (A) Dexamethasone treatment increased TEER values by 50% at day 8. Data represent mean ± SD, $n = 4$. $^{**}P < 0.01$ as compared to Z310 cells. (B). Dexamethasone treatment decreased [^{14}C]sucrose permeability coefficients $^{*}P < 0.05$ as compared to controls.

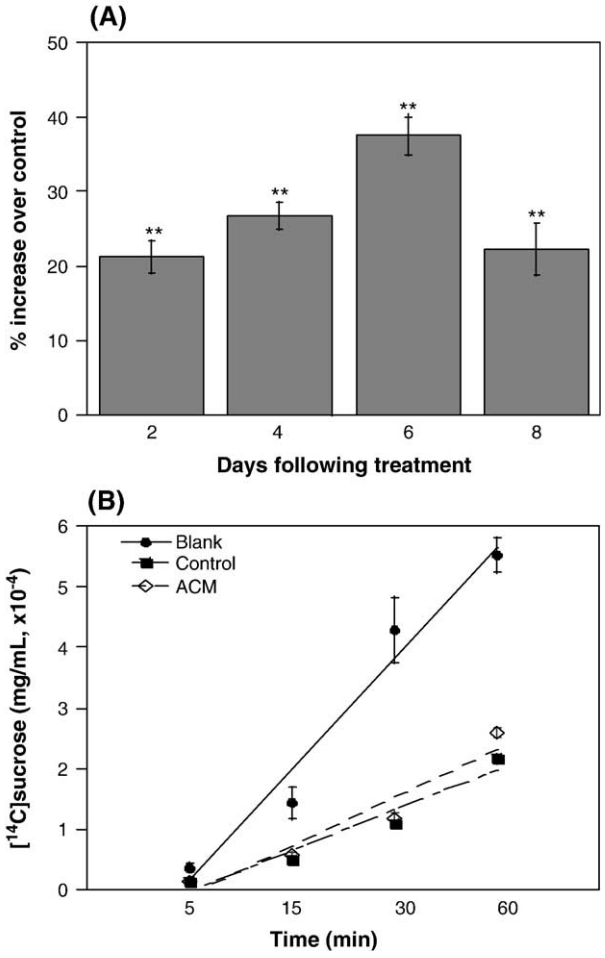

Fig. 5. Culture with astrocyte conditioned medium (ACM) significantly increased the TEER values (A), but did not affect [^{14}C]sucrose permeability (B). The ACM was collected from an established primary culture of rat astrocytes. Z310 cells grown on the collagen-coated polyester filter were cultured with the ACM 2 days after initial seeding in both the inner and outer chambers, for the duration of the study. Data represent mean ± SD, $n = 6$. **$P < 0.01$ as compared to normal growth medium.

0.27 Ω-cm^2). Studies on the paracellular permeability of [^{14}C]sucrose revealed that the [^{14}C]sucrose permeability coefficients from a 10-day culture were (2.59 ± 0.32) × 10^{-4} cm/min, in comparison to (5.98 ± 0.27) × 10^{-4} cm/min of the control group, about 231% less than the controls (Fig. 4B). These data suggest that treatment with dexamethasone increased TEER value and decreased sucrose leakage. While the parameters for the tightness of Z310 monolayer were greatly improved by dexamethasone treatment in comparison to primary plexus cell monolayer, the Z310 cell barrier remained to be less tighter than the barrier of MDCK cells (TEER: 231 ± 11 Ω-cm^2; [^{14}C]sucrose permeability coefficient: 2.4 ± 0.22 × 10^{-5} cm/min).

3.6. Treatment with modified culture media

Culture of Z310 cells with ACM significantly increased the TEER values of Z310 cell monolayer at all the tested time points (day 2, 4, 6, and 8, $P < 0.01$ as compared to controls). The maximal increase occurred at day 6, about 38% increase over the control values (Fig. 5A). However, ACM did not reduce the [^{14}C]sucrose permeability coefficient, even at day 8 following treatment (Fig. 5B). Following incubation with the SFM, the TEER value did not increase, instead it displayed a trend of decline over the time course of study (data not shown).

3.7. Treatment with EPA or EGF

Treatment of Z310 cells with either EPA or EGF showed the similar pattern of outcomes. Both compounds significantly increased the TEER values, with the maximal 38–40% increase over the controls (Fig. 6A and Fig. 7A). However, both compounds did not reduce the [^{14}C]sucrose permeability coefficients of Z310 cell monolayer in comparison to controls (Fig. 6B and Fig. 7B).

We also tested the combination treatment by culturing the cells with dexamethasone, EPA, or EGF in the ACM, combining dexamethasone with EPA or EGF in the growth medium, or mixing EPA and EGF in the growth medium, in

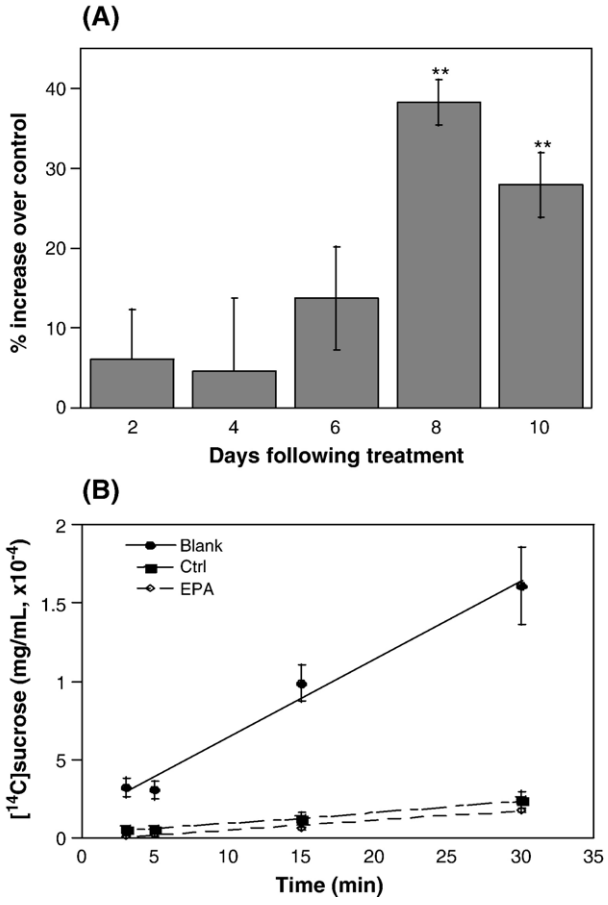

Fig. 6. The presence of EPA in the culture medium significantly increased the TEER values (A), but had no effect on [^{14}C]sucrose permeability (B). EPA (10 μM) was added to the culture medium of both chambers upon cell seeding and maintained throughout the entire experiment. Data represent mean ± SD, $n = 6$. **$P < 0.01$ as compared to controls.

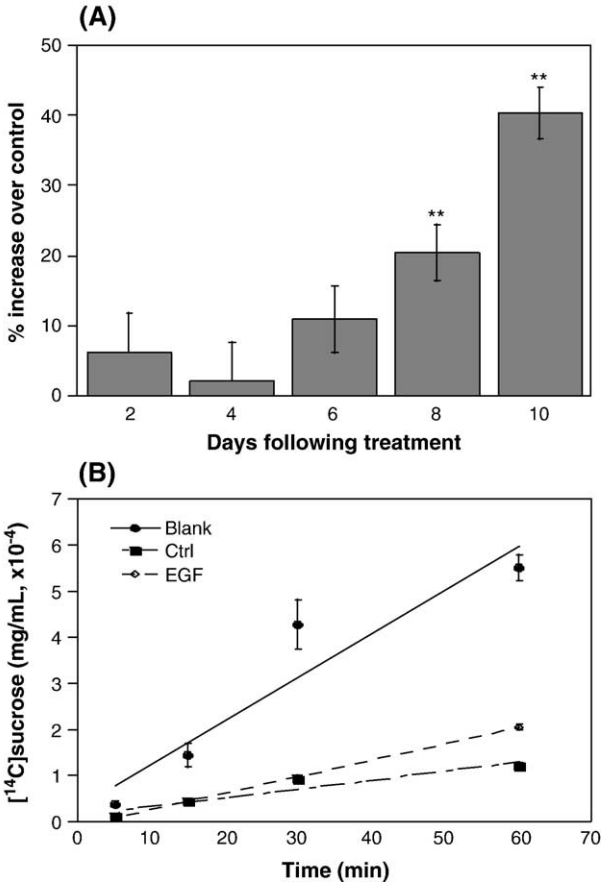

Fig. 7. Introduction of EGF (100 ng/mL) to the culture medium resulted in a significant increase in TEER values (A), but did not affect [^{14}C]sucrose permeability (B). EGF (100 ng/mL) was added to the culture medium of both chambers upon cell seeding and maintained throughout the entire experiment. Data represent mean ± SD, $n = 6$. $^{**}P < 0.01$ as compared to controls.

order to achieve a better tightness of Z310 barrier. However, none of these combination treatments yielded the higher TEER or lower sucrose permeability than did the treatment with dexamethasone alone (data not shown). Treatments with IL-15 (100 ng/mL) or GLA (10 μM) neither increased TEER values nor decreased [^{14}C]sucrose permeability (data not shown).

4. Discussion

Z310 cells possess the proteins that are essential to the formation of tight cell barrier. Our real-time RT-PCR analyses demonstrated that Z310 cells express mRNAs encoding tight junction proteins such as occludin, claudin-1, -2, -4, -8, ZO-1, -2, -3 and gap junction protein connexin-32. The presence of ZO-1 and claudin-1 in Z310 cells were further confirmed by confocal microscopic study. Interestingly, ZO-1 and claudin-1 showed a different pattern in their subcellular localization. ZO-1 staining was primarily located peripherally on the cell–cell contact sites, suggesting an immediate involvement of ZO-1 in the structure of tight junctions. Claudin-1, on the other hand, displayed a relatively diffusive staining pattern around the cell–cell contact, suggesting an extension of this protein from cell membrane into the cytoplasm. Previous reports in literature have identified a co-localization of ZO-1 and occludin along the apical–lateral surfaces of epithelia as well as inter-endothelial clefts of blood vessels [31]. However, unlike occludin, claudin-1 immunoreactivity has been shown to extend into the cytoplasm in endothelial cells [25] as well as in cornea epithelium [1]. Our confocal results confirm these observations, suggesting that Z310 cells possess the ability to form tight junctions between adjacent cells, which is the structural basis for brain barriers.

We used three benchmarks to justify the appropriateness to use Z310 cells as in vitro BCB barrier system: (1) Z310 cells should retain the essential morphology of the parent primary choroidal epithelial cells; (2) TEER values of Z310 cell monolayer should be comparable to the in vivo TEER values at the choroid plexus; and (3) [^{14}C]sucrose permeability coefficients of Z310 cell monolayer should be at least equal or less than the reported literature values. Our current results are in good agreement with our previous observation [47]; the Z310 cells displayed the same polygonal epithelial morphology as the primary choroidal epithelial cells by light microscopy.

When grown to the confluence on the membrane of the Transwell device, the TEER value and [^{14}C]sucrose permeability coefficient were about 61 Ω-cm^2 and 6×10^{-4} cm/min, respectively, at the current 257th passage. The [^{14}C]sucrose permeability coefficients determined from various in vitro brain barrier models vary between 2.7 and 41×10^{-4} cm/min [8,9,22,23]. The values obtained from Z310 cells fell within the literature values. However, the TEER values across the Z310 monolayer appeared to be less than those observed from live animals; in the latter case, the in vivo TEER values are in the range of 19–200 Ω-cm^2 [28,37,41]. Four approaches were then designed to improve the TEER of Z310 monolayer: (i) to change the growth medium condition by using SFM or ACM, (ii) to increase recruitment of TJ proteins to cell–cell contacts by introducing dexamethasone in the culture medium, (iii) to induce the production of TJ proteins by incubation with EPA, and (iv) to improve the cell differentiation with the application of EGF.

Astrocytes may contribute to the integrity of the BBB by inducing the formation of BBB in early development, by maintaining the intactness of BBB structure, and by participating in the transport of substances across the BBB. Several studies have shown that direct contact of endothelia with astrocytes or incubation with astrocyte conditioned media (ACM) can increase the expression of BBB-related proteins and enzymes, thus reducing the permeability of in vitro endothelial barriers [19,21]. It is suspected that soluble factors derived from astrocytes, e.g., TGF-β (transforming growth factor-β) [34], GDNF (glial

cell line-derived neurotrophic factor) [18], IL-6 (interleukin-6) and hydrocortisone [17] may play a role in regulation of tight junctions. Since the choroidal epithelial cells are bathed in the CSF to which the astrocytes contribute these and other yet undefined factors, we first tested the effect of ACM on Z310 barrier. Our results showed that incubation with ACM led to a significant increase in TEER values; however, it did not reduce the paracellular permeability of [^{14}C]sucrose. The discrepancy between increased TEER and unchanged sucrose permeability following ACM treatment suggests that some yet unknown factors in the ACM may reduce the ion conductance as reflected in the TEER value, while the proteins essential to the tight barrier may not be affected by ACM. Culturing the Z310 cells with serum free media using the recipes reported in literature [10,15] did not increase TEER, nor did they affect sucrose permeability.

Fatty acids are the major constituents of the phospholipid bilayer of the epithelial cell membrane and have been suggested to play a role in modulating the permeability of the epithelial cells [29]. Yamagata and his colleagues [42] recently reported that EPA and γ-linolenic acid (GLA), by modulating protein kinase C and tyrosine kinase activity, increased the expression of occludin mRNA in porcine brain capillary endothelial cells, leading to an increased TEER in their barrier model. Our data showed a significant increase of TEER following EPA treatment, which was consistent with reports by others. However, the lack of EPA's effect on [^{14}C]sucrose permeability limits the practical use of EPA as a tightness enhancer in our model system.

The epidermal growth factor (EGF), a 53-amino acid polypeptide, regulates the proliferation and differentiation of a variety of cell types, including polarized epithelial cells [2]. The epithelia express the receptor for EGF and related EGF binding ligands (i.e., EGFR, HER1, ErbB1), which belong to the superfamily of receptors with intrinsic tyrosine kinase activity [38]. Singh and Harris [30] found that EGF-induced EGFR activation in MDCK II cells had led to an inhibited claudin-2 expression, while it simultaneously induced the expression and cellular redistribution of claudin-1, -3, and -4, resulting in a 3-fold increase in TEER. Our data demonstrated that EGF did increase the TEER values in Z310 cells, but the magnitude of increase was much less than that in MDCK II cells as observed by Singh and Harris [30]. Our real-time RT-PCR revealed a relatively low expression of claudin-2 mRNA; earlier Western blot by this laboratory also failed to detect claudin-2 in Z310 cells (unpublished data). Thus, the difference in expression of claudin-2 between MDCK II cells and Z310 cells may explain the difference of these cell lines in response to EGF treatment. The increased TEER upon EGF treatment may be due to the fact that EGF may redistribute the transporters from the basolateral to the apical side and therefore increase the cell polarization, which in turn causes a slow entry of ions into cells and a subsequent increase of TEER. This mechanism needs to be further investigated.

Dexamethasone have been shown to increase the TEER values and decrease the paracellular permeability in non-transformed mouse mammary 31EG4 epithelial cells [40,43]. A multi-step cascade has been proposed for the mechanism of its action. Dexamethasone may up-regulate the expression of the Id-1 (DNA binding inhibitor-1), which is required for the steroid control of cell tight junction dynamics. Dexamethasone also facilitates the recruitment of the tight junction proteins (ZO-1 and occludin) and adherens junction proteins (E-cadherin and â-catenin) to the cell periphery at the sites of cell–cell contact. Our data clearly showed that treatment with dexamethasone not only increased the TEER values (about 107 Ω-cm^2) across Z310 cell monolayer, which was 50% higher than that of controls and was highly comparable to the in vivo TEER values (19–200 Ω-cm^2), but also decreased [^{14}C]sucrose permeability coefficients (about 2.6×10^{-4} cm/min), which was 231% less than that of controls and close to the lowest limit of the range reported in the literature (2.7×10^{-4} cm/min) ([8,9,22,23]. Thus, the presence of dexamethasone in the growth medium appears effectively to increase the tightness of the Z310 barrier.

Noticeably from the above studies, the tightness of the barrier was not exclusively reflected in the TEER values, but rather representative of a combination of both TEER and paracellular permeability. The TEER, which is the product of ion conductance (mainly by Na^+ in cell cultures) across the cell monolayer, represents the sum of two major electrical resistances, i.e., the paracellular resistance and the trans-cellular resistance; the former is associated with intercellular junctions and space, and the latter related to the activities of ion channels or pumps in both apical and basolateral cell membranes [6]. Thus, an increase in TEER does not simply imply an increase in tight intercellular junctions, but it could be due to an increased cell volume, which may obliterate the intercellular space [24]. The tightness of the barrier can also be described by the paracellular permeability of hydrophilic, uncharged paracellular diffusion markers such as sucrose or dextran. Our data showed a discrepancy between TEER values and sucrose permeability coefficients: treatment with ACM, EPA, and EGF all increased the TEER, but had no effect on sucrose paracellular permeability. This discrepancy is not uncommon in literature, as other investigators have also observed the similar results. For instance, when MDCK cells were cultured in the medium enriched with linolenic acid, the TEER was not affected, but the flux of dextran was increased by 6-fold [3]. Elevated expression of RhoA GTPase in MDCK cells increased TEER by 200%, while it increased (but not decreased) the mannitol permeability by 75% [16]. Thus, evaluation of the tightness of an in vitro monolayer barrier model must take into consideration both TEER and paracellular permeability of diffusion markers. Thus, although ACM, EPA, and EGF increase the TEER, the lack of their effect on sucrose permeability does not make them an ideal choice as the tightness enhancer of the in vitro BCB model system.

In summary, both real-time RT-PCR and confocal microscopic studies indicate that the Z310 cell line possesses the proteins essential to the formation of tight junctions. The Z310 cells at the current passage maintain the same polygonal choroidal morphology as the primary choroidal epithelial cells under microscope. When grown in a two-chamber Transwell system, the cells are capable of forming a cell monolayer with the TEER values and sucrose permeability comparable to those reported in literature. The presence of dexamethasone increases the TEER values and decreases the sucrose paracellular leakage of this Z310 cell-based BCB model. Thus, we recommend including dexamethasone in the growth medium for transport study at BCB. We also recognize that an increase of TEER does not necessarily parallel with a reduction of sucrose permeability, as evidenced in studies with ACM, EPA, and EGF. Thus, we caution that evaluation of the tightness of an in vitro barrier system should take into consideration both factors. The results presented in this report indicate that Z310 cell-based in vitro transport model is suitable for pharmacological and toxicological studies of drug and toxicants transport by the BCB in academia, clinical settings, pharmaceutical industry, and government regulatory agencies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2005.07.046.

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