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Original Article

Tightness comparison of ECV304 cells grown on two different types of transwell inserts

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Abstract: The blood-brain barrier (BBB), connected by interendothelial tight junctions, hinders the passage of substances into central nerve system. To mimic *in vivo* BBB conditions for pathological and pharmacological studies, the immortalized cell lines were developed. However, the brain endothelial cell lines often demonstrate insufficient tightness and thus the non-cerebral cell lines such as ECV304 cells are utilized as a BBB-mimicking tool. In this study, the expression of tight junction proteins occludin, claudin-5 and ZO-1 were confirmed in ECV304 cells by RT-PCR and Western-blot methods, and the immunofluorescence revealed the localization of three tight junction proteins along cell border, indicating the formation of tight junctions among ECV304 cells. The ECV304 monoculture model on polyethylene terephthalate (PET) membrane transwell inserts demonstrated higher TEER and lower permeability to hydrophilic marker lucifer yellow than that on polyester inserts. In addition, the permeability ratio of lipophilic marker propranolol to lucifer yellow was higher on PET model than that on polyester model. These results showed that the PET model of ECV304 cells developed tighter barrier and better discriminated between paracellular and transcellular flux than polyester model, suggesting its potential for passive transport studies.

Keywords: ECV304, blood-brain barrier, tight junction, transwell insert

Introduction

The blood-brain barrier (BBB), mainly consisted of brain microvascular endothelial cells, is connected by tight junctions and forms a restrictive physical barrier for molecular movements. It protects the brain from the xenobiotics and regulates the homeostasis of central nerve system. To investigate the physiology, pharmacology and biochemistry associated with BBB, *in vitro* cell-based models composed of primary brain endothelial cells and immortalized cell lines were established. Compared with primary cell culture, the immortalized cell lines are easy to grow and cost effective. The brain endothelial cell lines, including hCMEC/D3, bEnd3 and PBMEC/C1-2 and non-cerebral cell lines including MDCK-MDR1, CaCo-2 and ECV304 are developed for BBB research [1, 2]. The brain endothelial cell lines-based models usually form inadequate tightness barrier while epithelial cell lines like MDCK-MDR1 and CaCo-2 deliver morphological and functional features different from brain endothelium and show non-physically high expression of transporters [3]. ECV304, a human umbilical vein endothelial cell line but showing phenotypes similar to human bladder cancer cells [4], is inducible of tightness when co-cultured with rat glioma C6 cells. It developed tighter barrier than other endothelial cell lines such as RBE4, bEnd3 and bEnd5, and represents a promising BBB-mimicking cell line [5].

In this study, the expression of tight junction proteins occludin, claudin-5 and ZO-1 in ECV304 cells were identified through the methods of RT-PCR, Western blot and immunofluorescence assay. The ECV304 monoculture models on polyester and polyethylene terephthalate (PET) membrane transwell inserts were established. The functional tightness of the models was assessed by measurement of transendothelial electrical resistance (TEER)

Table 1. The primers used in this study

Gene	Sense primer sequence	Antisense primer sequence
GADPH	5'-CTATAAATTGAGCCCGCAGCC-3'	5'-GCGCCCAATACGACCAAATC-3'
Occludin	5'-CTCTCGGGCCGCAACATC-3'	5'-CTCCCTCGGTGACCAATTCA-3'
Claudin-5	5'-CCGCTTTTTGCCAGAGACTCA-3'	5'-AGCACTGTCTCTCTCATCCC-3'
ZO-1	5'-TGGCACATCAGCACGATTTC-3'	5'-CAAACAGACCAAGCCAGCAC-3'

and permeability to hydrophilic marker lucifer yellow. In addition, the permeation of lipophilic marker propranolol was measured to evaluate the discriminative brain penetration of the models. The aim of the study is to establish a tight model based on ECV304 cell line for drug passive transport studies.

Materials and methods

Materials

ECV304 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Medium 199 (M199), fetal bovine serum (FBS), penicillin-streptomycin solution, trypsin (0.25%)-EDTA (0.02%) solution and Hank's balanced salt solution (HBSS) were purchased from Hyclone (Logan, UT, USA). Lucifer yellow was purchased from Sigma Aldrich (St. Louis, MO, USA). Propranolol was obtained from Macklin Inc. (Shanghai, China). The following antibodies were purchased from Abcam (Cambridge, UK): rabbit claudin-5 antibody and Alexa Fluor 488 conjugated goat anti-rabbit IgG. Rabbit occludin and ZO-1 antibody were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Mouse GADPH antibody and HRP-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Applygen Technologies Inc. (Beijing, China). The PVDF membrane and chemiluminescent HRP substrate were purchased from EMD Millipore (Billerica, MA, USA). Lastly, the polyester (#3470) and PET (#Falcon 353095) membrane 24-well transwell inserts (pore size: 0.4 μM, diameters: 6.5 mm) were obtained from Corning Incorporated, (Corning, NY, USA).

Cell culture

The ECV304 cells were cultured in M199 medium supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. The confluent cells were passaged by the trypsin (0.25%)-EDTA (0.02%) solution at a split ratio of 1:5.

RT-PCR

Total RNA was extracted from bEnd3 cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocols, and quantified with an UV spectrophotometer (Biospec nano, Shimadzu Corporation, Kyoto, Japan). A total of 1 μg RNA was reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Shiga, Japan), according to the manufacturer's protocols. The PCR amplification was performed using the SYBR Premix Ex Taq II Kit (Takara), according to the manufacturer's protocols, in the Takara system, under the following thermocycling conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 30 sec. The gene primer sequences were shown in **Table 1**. The PCR products were electrophoresed on 1.2% agarose gels and visualized by UV light in the presence of ethidium bromide.

Western blot

The harvested ECV304 cells were lysed using a RIPA buffer containing the protease inhibitor. The protein concentration was determined by BCA protein assay Kit (Applygen). A total of 30 μg of denatured protein was loaded on the 10% SDS-PAGE gel for electrophoretic separation followed by a transfer to the PVDF membrane. The membrane was incubated with 5% powdered skimmed milk in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature to block the non-specific sites. After three washes with PBST, the incubation of the membrane with primary antibodies for GADPH (dilution, 1:2,000), occludin, claudin-5 and ZO-1 (dilution, 1:1,000) were performed overnight at 4°C. Following incubation with HRP-conjugated secondary antibody (dilution, 1:5,000) lasted for 1 h at room temperature, the bands were visualized through chemiluminescence detection in a FluorChem® FC2 system (Cell Biosciences, Inc., Santa Clara, CA, USA).

Immunocytochemistry

The confluent ECV304 cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After three washes with PBS, the cells were blocked with 5% normal goat

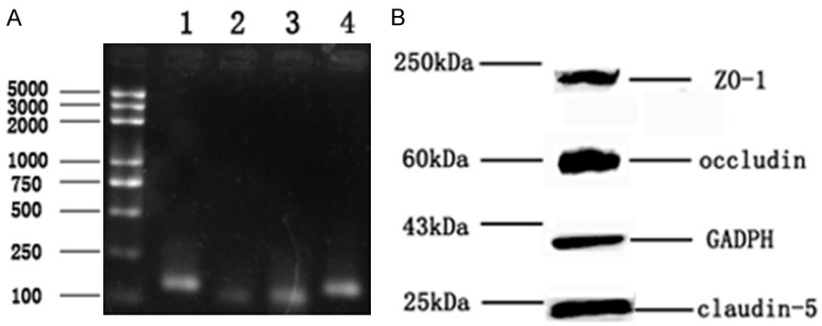

Figure 1. Expression of tight junction proteins occludin, claudin-5 and ZO-1 in ECV304 cells. A. The mRNA expression of GADPH (lane 1), occludin (lane 2), claudin-5 (lane 3) and ZO-1 (lane 4) in ECV304 cells. B. The protein expression of GADPH (37 kDa), occludin (59 kDa), claudin-5 (24 kDa) and ZO-1 (230 kDa) in ECV304 cells.

serum (Applygen Technologies Inc., Beijing, China) containing 0.1% Triton X-100 for 30 min and immunoblotted with primary antibodies for occludin, claudin-5 and ZO-1 (dilution, 1:100) overnight at 4°C. After three washes with PBS, the cells were incubated with Alexa Fluor 488 conjugated secondary antibody (dilution, 1:500) for 1 h at room temperature and then stained with DAPI. The cells were mounted using fluorescent mounting medium (ZSGB-BIO, Beijing, China) and the images were visualized using a ZEISS LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Transport studies

For the generation of monoculture models, the ECV304 cells were seeded at a density of 5 × 10^4/cm^2 on the upper compartment of the polyester or PET membrane transwell inserts. The cells were maintained at 37°C and the medium was changed every 48 h post-seeding. After 7 days of cell incubation, the TEER in each insert was measured using the EVOM epithelial voltohmmeter (World Precision Instrument, USA). The TEER was normalized by subtracting the resistance of blank inserts and shown as Ω·cm^2. For the transport studies, the medium in the upper compartment was replaced by HBSS containing tested compounds (50 μM for lucifer yellow, 20 μM for propranolol). At indicated time points (15, 30, 45, 60 min), the sample was taken from the lower compartment and supplemented with an equal volume of fresh HBSS. The concentration of lucifer yellow was analyzed by a PerkinElmer EnSpire Multimode Plate Reader (excitation: 430 nM; emission: 540 nM) while that of propranolol was measured by the LC-MS/MS method. The permeability coefficient P_e was calculated by the following equation: $P_e = S/(C_0A)$ where C_0 is the initial drug concentration at the upper compartment, A is the membrane surface area and S is the slope of a plot of the cumulative drug mass at the lower compartment versus time. The permeability coefficient of the cell layers P_e (cell) was calculated by the following equation: $1/P_e$ (cell) = $1/P_e$ (total) - $1/P_e$ (blank).

Statistical analysis

The values between groups were compared by one-way analysis of variance followed by Tukey test using SPSS (version 19.0; SPSS Inc., Chicago, IL, USA). The significant difference was determined at $p < 0.05$.

Results

Expression of tight junction proteins in ECV304 cells

As shown in **Figure 1**, the mRNA and protein expression of tight junction proteins occludin, claudin-5 and ZO-1 were confirmed in ECV304 cells. The immnofluorescence revealed their localization on cell plasma membrane. The staining of ZO-1 gave distinct strands along cell border while those of occludin and claudin-5 were diffuse and weak (**Figure 2**).

Functional analysis of ECV304 monoculture models on PET and polyester membrane transwell inserts

The ECV304 monoculture model on the PET membrane transwell insert achieved significantly higher TEER and lower permeability of lucifer yellow than that on polyester inserts (TEER: 68.6 ± 10.1 Ω·cm^2 vs 28.3 ± 2.1 Ω·cm^2; P_e: 4.1 ± 1.1 × 10^{-6} cm/s vs 14 ± 2 × 10^{-6} cm/s). The permeability coefficients of propranolol in PET and Polyester model of ECV304 cells were 26.7 ± 2.6 × 10^{-6} cm/s and 27.7 ± 0.22 × 10^{-6} cm/s, respectively (**Figure 3**). Consequently,

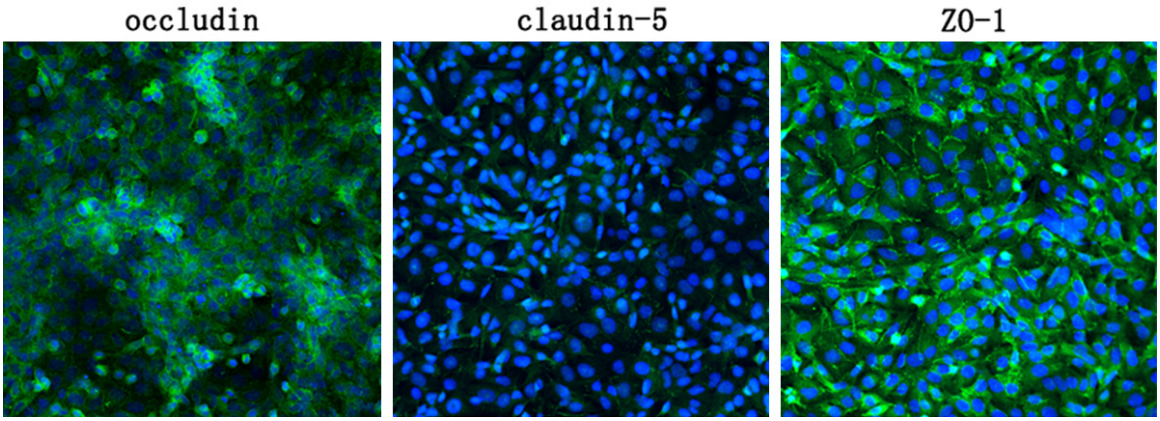

Figure 2. Immunofluorescent staining of tight junction proteins occludin, claudin-5 and ZO-1 in ECV304 cells. The immnofluorescence of occludin, claudin-5 and ZO-1 demonstrated their localization along cell margin. The staining of ZO-1 revealed the distinct bands on cell border while those of occludin and claudin-5 were diffuse and weak. The confocal images were acquired at 20 × magnification.

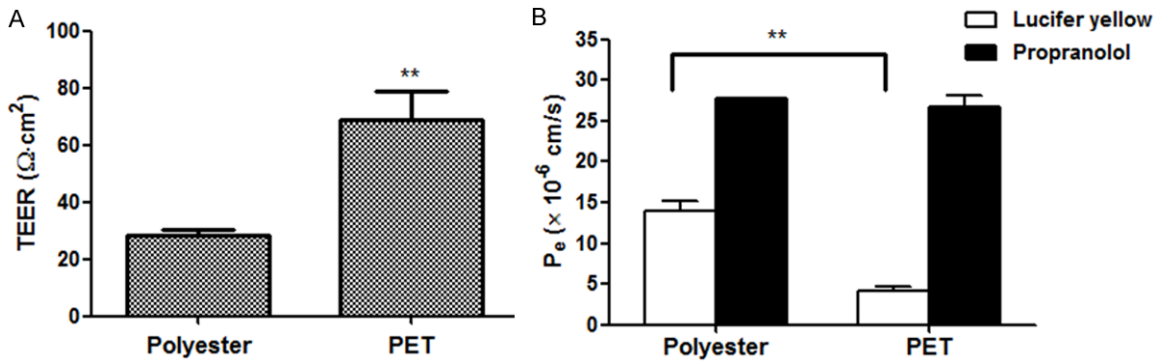

Figure 3. The TEER (A) and the permeability to lucifer yellow and propranolol (B) of ECV304 monoculture models on PET and polyester membrane transwell inserts. The ECV304 cells were seeded at a density of 5 × 10^4/cm^2 on the upper compartment of PET or polyester membrane transwell inserts and incubated for 7 days to generate the monoculture models. The PET model of ECV304 cells revealed significantly higher TEER and lower lucifer yellow permeability than polyester model. In addition, the PET model showed a higher permeability ratio of propranolol to lucifer yellow than polyester model. Data represent means ± SD (n = 3). $^{**}p < 0.01$.

the permeability ratio of propranolol to lucifer yellow on PET model was 6.5 vs that of 2 on polyester model.

Discussion

The interendothelial tight junction is the key feature of the BBB, and it forms a restrictive barrier for paracellular permeability. However, the immortalized brain endothelial cell lines-based BBB models often reveal inadequate tightness and thus the non-cerebral cell lines such as ECV304 cells are applied for BBB modeling. Here we identified the expression of tight junction proteins occludin, claudin-5 and ZO-1 in ECV304 cells and compared the functional tightness of ECV304 monoculture models on PET and polyester membrane transwell inserts.

The tight junction is a complex of multiple proteins including junction adhesion molecules, occludin, claudins and zonula occluden (ZO-1, ZO-2 and ZO-3). Occludin and claudin-5 are transmembrane proteins, and they interact with the corresponding proteins on adjacent endothelial cells to seal the intercellular cleft, resulting in the limited paracellular permeability. ZO-1 is a cytoplasmic protein, and it mediates the communication between transmembrane proteins and actin cytoskeleton, regulating the function of barrier integrity. The loss of these proteins may be associated with disrupt-

ed barrier function or BBB breakdown [6]. In this study, the mRNA and protein expression of occludin, claudin-5 and ZO-1 were confirmed in ECV304 cells. The immunofluorescence of three proteins demonstrated their localization on cell membrane, suggesting the tight junction formation among ECV304 cells. However, the staining of occludin and claudin-5 were weak and diffuse, indicating that the tightness of the cell line is not as sufficient as that of primary culture of brain endothelial cells which showed evident immunofluorescent bands along cell border [7]. The strategies of adding enhancers such as hydrocortisone or CPT-cAMP or growing in glial cells-conditioned medium deserved to be implemented to elevate the tightness of ECV304 monolayers [8, 9].

The BBB models could be categorized into the static models and dynamic models. The static model using transwell insert filter is the most commonly utilized tool for BBB research today [10]. Since the availability of a variety of transwell inserts different in size and membrane materials, the insert type may influence the model tightness. It is reported that the mouse brain endothelial cells grown on PET membrane transwell inserts developed tighter barrier than those on polycarbonate membrane inserts [11]. The functional tightness of BBB models is usually assessed by two indicators: TEER and permeability to polar molecules. The TEER reflects the resistance of the barrier to ion movement. However, the TEER is variable among laboratories due to difference in instrumentation and experimental conditions such as temperature and ion composition of the medium. More importantly is the tracer permeability [12]. In this study, the ECV304 monoculture model on PET membrane transwell inserts achieved higher TEER and lower permeability to hydrophilic marker lucifer yellow than that on polyester inserts. The TEER in ECV304 monoculture model on PET inserts (68.6 $\Omega \cdot cm^2$) was comparable with that reported before (62 $\Omega \cdot cm^2$) while the lucifer yellow permeability ($4.1 \pm 1.1 \times 10^{-6}$ cm/s) was lower than that in published ECV304 (10.4×10^{-6} cm/s) and hCMEC/D3 (22.2×10^{-6} cm/s) models [13, 14]. In addition, the permeability of lipophilic marker propranolol was determined to evaluate the discriminative brain penetration of the ECV304 models. The PET model revealed higher permeability ratio of propranolol to lucifer yellow than polyester model (6.5 vs 2) while this value is documented as 3.4 in an hCMEC/D3 model [14].

In summary, the ECV304 cells formed intercellular tight junctions. The ECV304 monoculture model on PET membrane transwell inserts demonstrated tighter barrier and better discrimination between paracellular and transcellular flux than polyester model, indicating its potential to be explored as an *in vitro* tool for BBB passive transports studies.

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Disclosure of conflict of interest

None.

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