Paracellular Barrier and Tight Junction Protein Expression in the Immortalized Brain Endothelial Cell Lines bEND.3, bEND.5 and Mouse Brain Endothelial Cell 4

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The blood–brain barrier (BBB) is formed by brain endothelial cells. Many immortalized brain endothelial cell lines have been established; these have been used as *in vitro* BBB models. The aim of the present study was to assess the paracellular barrier properties of the immortalized mouse brain endothelial cell lines bEND.3, bEND.5 cells, and mouse brain endothelial cell 4 (MBEC4), and those of the primary mouse brain endothelial cells pMBECs. bEND.3 cells showed low permeability to sodium fluorescein and obvious staining of tight junction proteins (claudin-5, occludin and ZO-1) similar to pMBECs; these barrier properties of MBEC4 and bEND.5 cells were low. In addition, bEND.3 cells expressed the highest level of claudin-5 among all cells. These results suggest that bEND.3 cells are a convenient and useful model for evaluating BBB function, especially the paracellular barrier.

Key words blood–brain barrier; tight junction; brain endothelial cell

The blood–brain barrier (BBB) consists of the brain endothelial cells of cerebral capillaries.[1] These endothelial cells express specific ion and peptide transporters, which form a transcellular barrier; they also form a paracellular barrier owing to the presence of tight junctions between adjacent endothelial cells.[2] The BBB contributes to homeostasis of the central nervous systems (CNS) by restriction of the transport of substances between brain and blood. Reliable *in vitro* BBB models are required for evaluations of the BBB function, especially the paracellular barrier.

Recently, many immortalized brain endothelial cell lines have been established and employed as *in vitro* BBB models.[3] Among the many immortalized brain endothelial cell lines, bEND.3 cells and bEND.5 cells are available from commercial cell banks such as the European Collection of Cell Cultures (ECACC) or the American Tissue Culture Collection (ATCC). Both cell lines were established using isolated mouse brain endothelial cells; the BBB functions of these cell lines were evaluated compared with those of primary brain endothelial cells or the same cell lines grown in the other culture conditions.[4–7] However, there are no comparative studies on BBB functions among several immortalized brain endothelial cell lines.

In this study, we performed comparative studies of the immortalized mouse brain endothelial cell lines bEND.3, bEND.5 and MBEC4 cells, as well as pMBECs. We selected these three mouse cell lines to compare with pMBECs established in many laboratories. MBEC4 cells were established using isolated mouse brain microvascular endothelial cells and showed the highly specialized characteristics of brain microvascular endothelial cells.[8] Tight junction proteins (claudin-5, occludin and ZO-1) are essential for brain endothelial cells to induce and maintain the tightness of tight junctions (the paracellular barrier).[9–11] Therefore, we examined the expression levels and distribution of these tight junction proteins and paracellular permeability in the cell lines MBEC4, bEND.3 and bEND.5 cells, and pMBECs.

MATERIALS AND METHODS

Animals ICR mice were obtained from Kyudo (Kumamoto, Japan). Experiments were carried out in compliance with the guidelines stipulated by the Animal Care and Use Committee of Fukuoka University.

Isolation and Culture of pMBECs The protocol for isolating pMBECs and growing them was modified from that of Banks *et al.*[12] Brains from anesthetized 7–8-week-old ICR mice were cleaned of the meninges and homogenized using a hand-held scalpel. Homogenates were digested in a collagenase solution (1 mg/mL collagenase type II containing 15 μg/mL DNase I) at 37°C for 30 min. Neurons, astrocytes and other cells were removed by centrifugation (1000×g for 20 min) in Dulbecco's modified Eagle's medium (DMEM) containing 20% bovine serum albumin(BSA). The partially purified cell mixture was digested again (1 mg/mL collagenase/dispase containing 6.7 μg/mL DNase I) at 37°C for 30 min. Final purification of endothelial cells was obtained by differential centrifugation on a 33% Percoll gradient (GE Healthcare, Buckinghamshire, U.K.) at 1000×g for 10 min. pMBECs were placed in fibronectin- and collagen IV (both 0.1 mg/mL; Sigma-Aldrich, St. Louis, MO, U.S.A.)-coated culture dishes. Cultures were maintained for 2 d in DMEM/F12 supplemented with 10% plasma-derived serum, basic fibroblast growth factor (1.5 ng/mL), heparin (100 μg/mL), insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL), gentamicin (50 μg/mL), and puromycin (4 μg/mL) (pMBECs medium I) at 37°C in a humidified atmosphere of 5%CO_2–95%air. On the third day, pMBECs were cultured in new medium containing all the components of pMBECs medium I except for puromycin (pMBECs medium II). Five or

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6 d later, pMBECs were seeded on fibronectin- and collagen IV-coated 6-well plates at a density of 20800 cells/cm^2 for immunoblot analysis. For permeability test and immunocytochemical study, these cells were seeded onto the insides of fibronectin- and collagen IV-coated polyester membranes of Transwell-Clear inserts (diameter 6.5 mm, 0.4-μm pore size; Corning, Acton, MA, U.S.A.) placed in the wells of a 24-well culture plate (33000 cells/cm^2). Then pMBECs were cultured in pMBECs medium II containing 500 nM hydrocortisone for 48–60 h before experiments.

Immortalized Mouse Brain Endothelial Cell Line Culture MBEC4 cells were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation.[8] bEND.3 cells were obtained from the ATCC (Manssas, VA, U.S.A.). MBEC4 cells and bEND.3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin. bEND.5 cells were obtained from the ECACC (Salisbury, U.K.) and were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acid (10 mM), 1% sodium pyruvate (100 mM), 2% L-glutamine (200 mM), 100 units/mL penicillin and 100 μg/mL streptomycin. All cells were cultured at 37°C in a humidified atmosphere of 5%CO_2–95%air. All cells were seeded on fibronectin- and collagen IV-coated plates and Transwell-Clear inserts in the same manner as pMBECs. All cells were cultured in medium containing 500 nM hydrocortisone for 48–60 h before experiments.

Paracellular Transport of Sodium Fluorescein Endothelial paracellular barrier function was evaluated by measuring the permeability of cells to sodium fluorescein (Na-F), following a previously described protocol.[13] To initiate transport experiments, the medium was removed and cells were washed three times with Krebs–Ringer buffer (KRB; 118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4). KRB containing 100 μg/mL Na–F was loaded onto the luminal side of the insert. Samples were removed from the abluminal chamber at 30, 60, 90, and 120 min and immediately replaced with fresh KRB. The concentration of Na–F was determined using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm; CytoFluor Series 4000; PerSeptive Biosystems, Framingham, MA, U.S.A.). The permeability coefficient was calculated as previously described.[13]

Immunoblot Analysis Cells cultured on fibronectin- and collagen IV-coated 6-well plates were grown to 90–100% confluence. Cell lysates containing 10 μg of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and semidry blotting was performed at 2 mA/cm^2 (Bio-RAD, Hercules, CA, U.S.A.). The blots were blocked with blocking reagent, Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated with primary antibody [mouse anti-ZO-1 (1 : 1000; Zymed Laboratories, South San Francisco, CA, U.S.A.), mouse anti-occludin (1 : 1000; Zymed Laboratories), rabbit anti-claudin-5 (1 : 1000; Zymed Laboratories), or rabbit anti-beta-actin (1 : 5000, Abcam, Cambridge, U.K.)], followed by sheep anti-mouse immunoglobulin G (IgG) antibody-horse-radish peroxidase (HRP) conjugate (1 : 10000, GE Healthcare), or donkey anti-rabbit IgG antibody-HRP conjugate (1 : 10000, GE Healthcare). The blots were visualized using an ECL Advance Western Blotting Detection kit (GE Healthcare) and analyzed using a Fluor chem imaging system (Alpha Innotech, Santa Clara, CA, U.S.A.).

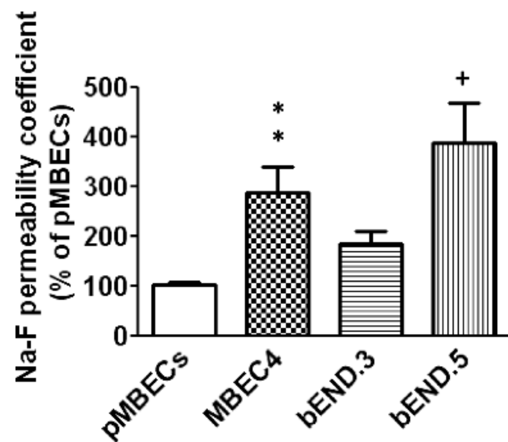

Fig. 1. Paracellular Barrier in pMBECs and Three Immortalized Brain Endothelial Cell Lines

Permeability coefficients were estimated by measuring the amounts of Na–F that passed through cell layers. Data are expressed as a percentage of control values (pMBECs). Values are means±S.E. (each group n=9). ** p<0.01 and † p<0.001, significantly different from pMBECs.

Immunocytochemical Studies After the Na–F permeability study, cells on Transwell inserts were washed five times with PBS, and fixed with 95% ethanol–5% acetic acid for 10 min at –20°C (ZO-1) or with ethanol for 1 min at room temperature (claudin-5 and occludin). Cells were blocked with Blocking One, and incubated with primary antibody [rabbit anti-ZO-1 (1 : 100), mouse anti-claudin-5 (1 : 100), or anti-occludin (1 : 100)] overnight at 4°C (all purchased from Zymed Laboratories). Cells were then incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG antibody for ZO-1 (1 : 50, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) or Cy3-labeled anti-mouse IgG antibody for claudin-5 and occludin (1 : 50, Jackson ImmunoResearch laboratories, Inc.) for 1 h at room temperature. A mesh of Transwell insert was cut off and was mounted in Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, U.S.A.) and then inspected by fluorescence microscopy (Keyence BZ-8000, Keyence Corporation, Osaka, Japan).

Statistical Analysis Data from the Na–F transport assay were evaluated for statistical significance using the Kruskal–Wallis test followed by Dunn's multiple comparison test. Data from the immunoblot analysis were evaluated for statistical significance using one-way analysis of variance followed by Bonferroni's multiple comparison test. The criterion for statistical significance was p<0.05. Data are presented as means± S.E.

RESULTS AND DISCUSSION

As shown in Fig. 1, MBEC4 and bEND.5 cells showed 2.9- and 3.9-fold higher permeability coefficients of Na–F than pMBECs, respectively. There was no significant difference in the permeability coefficients between bEND.3 cells and pMBECs; these values for bEND.3 cells were the lowest among those for the three immortalized cell lines. These results suggest that bEND.3 cells have a potent paracellular barrier property. Restrictiveness of the paracellular pathway is maintained by tight junction proteins including

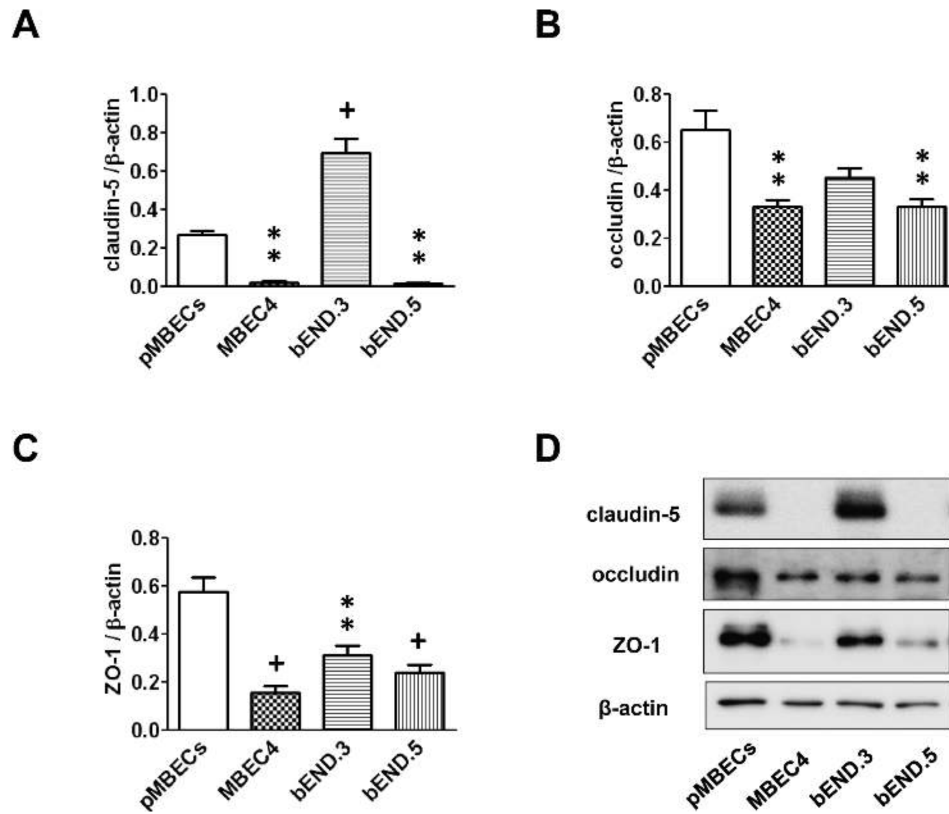

Fig. 2. Expression Levels of Tight Junction Proteins in pMBECs and Three Immortalized Brain Endothelial Cell Lines

Densitometric analysis of immunoblots showing claudin-5 (A), occludin (B) and ZO-1 (C). Representative immunoblots showing the expression of each tight junction protein (D). Band intensities were quantified by scanning densitometry (using β-actin as a loading control). Values are means±S.E. (each group n=5). ** $p<0.01$ and $^{+}p<0.001$, significantly different from pMBECs.

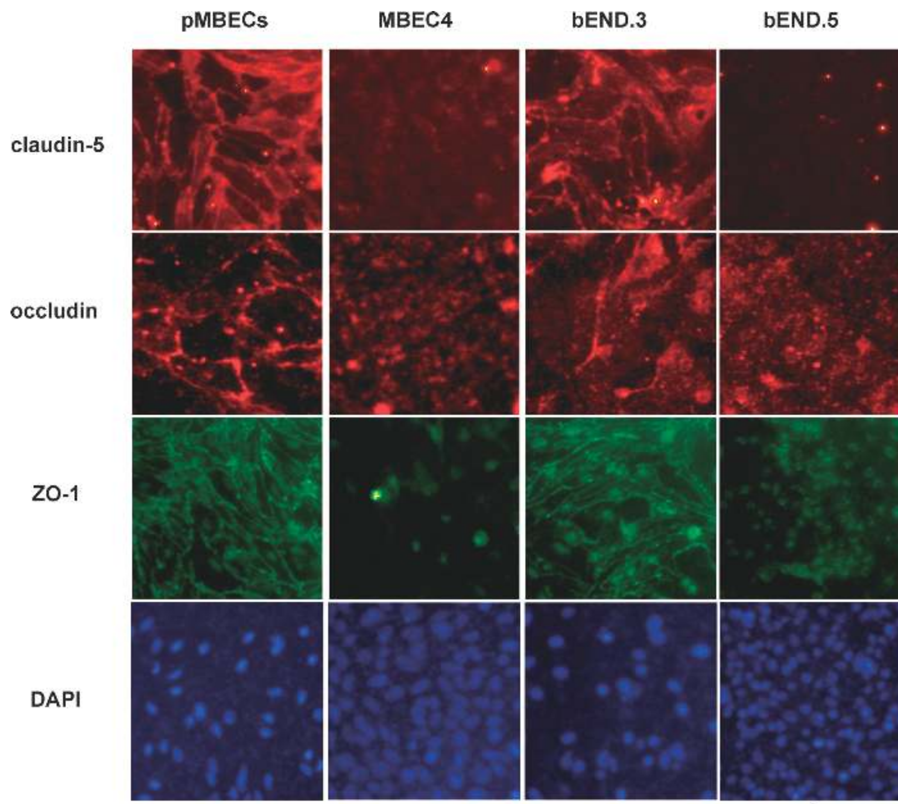

Fig. 3. Distribution of Tight Junction Proteins in pMBECs and Three Immortalized Brain Endothelial Cell Lines

Representative photographs showing immunofluorescent staining for claudin-5, occludin, ZO-1 and DAPI. Scale bar: 100 μm.

the transmembrane proteins occludin and claudin, and the cytoplasmic scaffolding proteins ZO-1.[9–11] Although previous studies have already reported that bEND.3 cells express some important tight junction proteins,[4,5] there has been no comparative study on the expression levels of tight junction proteins among immortalized brain endothelial cells.

The present immunoblot analysis showed that claudin-5 expression levels in MBEC4 and bEND.5 cells were significantly lower than those in pMBECs, while bEND.3 cells showed 2.6-fold higher levels of claudin-5 than did pMBECs (Fig. 2A). Occludin levels in MBEC4 cells and bEND.5 cells were significantly lower than those in pMBECs, but those in bEND.3 cells were not significantly different from those in pMBECs (Fig. 2B). ZO-1 levels in the three immortalized cell lines were significantly lower than those in pMBECs (Fig. 2C). Next, immunocytochemical observations revealed that claudin-5, occludin and ZO-1 immunoreactivities were strongly expressed in the vicinity of cell borders in pMBECs, showing a linear shape along cell junctions (Fig. 3). bEND.3 cells also exhibited obvious staining for claudin-5 and ZO-1, which were distributed along the intercellular junctions, while staining for occludin was moderate (Fig. 3). Conversely, MBEC4 cells and bEND.5 cells showed an indistinct and discontinuous distribution of tight junction proteins along cell borders (Fig. 3).

There is a report suggesting that claudin-5 is largely responsible for the paracellular barrier.[9] bEND.3 cells expressed higher levels of tight junction proteins, especially claudin-5, than MBEC4 and bEND.5 cells (Fig. 2). The immunostaining of tight junction proteins in bEND.3 cells showed marked expression and distribution of these proteins along the intercellular junctions (Fig. 3). These results show that the strong paracellular barrier in bEND.3 cells can be attributed to the high expression levels and efficient distribution of tight junction proteins, especially claudin-5. Therefore, bEND.3 cells are highly likely to be a convenient and useful model for evaluating BBB function, especially the paracellular barrier.

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