Establishment and Characterization of Human Peripheral Nerve Microvascular Endothelial Cell Lines: A New *in vitro* Blood-Nerve Barrier (BNB) Model

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ABSTRACT. The blood-nerve barrier (BNB) is a highly specialized unit that maintains the microenvironments of the peripheral nervous system. Since the breakdown of the BNB has been considered a key step in autoimmune neuropathies such as Guillain-Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy, it is important to understand the cellular properties of the peripheral nerve microvascular endothelial cells (PnMECs) which constitute the BNB. For this purpose, we established an immortalized cell line derived from human PnMECs. The human PnMECs were transduced with retroviral vectors encoding the temperature-sensitive SV40 large T antigen and human telomerase. This cell line, termed FH-BNB, showed a spindle fiber-shaped morphology, expression of von Willebrand factor and uptake of acetylated low density lipoprotein. These cells expressed tight junction proteins including occludin, claudin-5, ZO-1 and ZO-2 at the cell-cell boundaries. P-glycoprotein and GLUT-1 were also detected by a Western blot analysis and the cells exhibited the functional expression of p-glycoprotein. In addition, transendothelial electrical resistance experiments and paracellular permeabilities of sodium fluorescein and fluorescein isothiocyanate-labeled dextran of molecular weight 4 kDa across these cells demonstrated that FH-BNBs had functional tight junctions. These results indicated that FH-BNBs had highly specialized barrier properties and they might therefore be a useful tool to analyze the pathophysiology of various neuropathies.

Key words: blood-nerve barrier/endothelial cell lines/tight junction/transporter/claudin-5

Introduction

The blood-nerve barrier (BNB) serves as a functional and structural unit protecting the nervous system from circulating blood (Poduslo *et al.*, 1994). The BNB comprises the endoneurial microvasculature and the innermost layers of the perineurium. The perineurium is composed of flattened cells derived from fibroblasts, and these cells form several layers (Weerasuriya, 2005). Additionally, both sides of the perineurium are surrounded by basement membrane, and

the perineurium is thought to be a specialized type of connective tissue (Weerasuriya, 2005). Many studies (Soderfeldt, 1974; Boddington, 1984; Weerasuriya and Rapoport, 1986; Weerasuriya *et al.*, 1989) have demonstrated that the perineurium shows much lower permeability against many substances than endoneurial microvessels. Hence, the peripheral nerve microvascular endothelial cells (PnMECs) constituting the microvessels in the endoneurium can be considered to be the main interface between the blood and peripheral nerves. It has recently been reported that disruptions and permeability changes in the endoneurial microvessels play a crucial role in many autoimmune disorders of the peripheral nervous system including Guillain-Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), and paraneuritic neuropathy (Lach *et al.*, 1993; Kanda *et al.*,

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1994, 2000, 2004). Because PnMECs constitute the bulk of the BNB, *in vitro* studies using cultured PnMECs could be very useful to obtain a better understanding of the pathophysiology of these peripheral nerve disorders. However, only a few *in vitro* BNB models have been reported so far (Argall *et al.*, 1994; Kanda *et al.*, 1997; Iwasaki *et al.*, 1999; Sano *et al.*, 2007). This is probably due to the technical difficulties in isolating PnMECs from a peripheral nerve.

Transgenic rats harboring a temperature-sensitive SV40 large T-antigen gene (tsA58) have recently been developed as a source of conditionally immortalized cell lines (Takahashi *et al.*, 1999). Cultured cells derived from these animals can be easily immortalized by activating the tsA58 gene at a permissive temperature of 33°C and can have *in vivo* functions at a non-permissive temperature of 37 or 39°C (Takahashi *et al.*, 1999; Hosoya *et al.*, 2000). Human PnMECs expressing the tsA58 gene could have the potential to be used in studies elucidating their role in many diseases affecting the human peripheral nervous system. Recently, we established a conditionally immortalized PnMEC cell line derived from a human sciatic nerve and analyzed the effects of hydrocortisone on these endothelial cells (Kashiwamura *et al.*, 2011). However, the barrier properties of this cell line have not been fully characterized.

The purpose of the present study was to establish a conditionally immortalized human peripheral nerve microvascular endothelial cell line. O'Hare *et al.* reported that transgenic expression of the catalytic subunit of human telomerase (hTERT) in combination with the temperature-sensitive SV40 large T-antigen is necessary to immortalize human endothelial cells of different peripheral organs (O'Hare *et al.*, 2001). We therefore immortalized human PnMECs through coexpression of hTERT and tsA58 using retroviral vector systems, and investigated the barrier-specific properties of this novel *in vitro* BNB model.

Materials and Methods

Materials

Polyclonal anti-occludin, anti-Zonula occludens-1 (ZO-1), and anti-claudin-5 antibodies were obtained from Zymed (San Francisco, CA, U.S.A.). The monoclonal anti-p-glycoprotein (p-gp) antibody was obtained from Signet Laboratories (Dedham, MA, U.S.A.). The polyclonal anti-Zonula occludens-2 (ZO-2) and anti-von Willebrand factor (vWF) antigen antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). The polyclonal anti-human catalytic unit of telomerase (hTERT) antigen antibodies was obtained from Rockland Immunochemicals, Inc (Gilbertsville, PA, U.S.A.). The monoclonal anti-SV40 T antigen antibody was purchased from Calbiochem (Darmstadt, Germany). Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Zymed. The retrovirus vector pDON-AI was from TAKARA Bio Inc (Otsu, Japan). Rhodamine123, sodium fluores-

cein and FITC-labeled dextran of molecular weight 4 kDa were purchased from Sigma (St. Louis, MO, U.S.A.). Calcein-acetoxymethylester (calcein-AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Verapamil was purchased from WAKO (Osaka, Japan). Human umbilical vein endothelial cells (HUVECs), HEK293 cells and HL-60 cells were obtained from Japan Health Sciences Foundation (Osaka, Japan).

Culture media

The endothelial cell (EC) growth medium contained EBM-2 medium (Cambrex, Walkersville, MD, U.S.A.) supplemented with 20% fetal bovine serum (FBS) (Highclone, Logan, UT, U.S.A.), EGM-2 MV (Cambrex), 100 U/ml penicillin (Sigma), 100 µl/ml streptomycin (Sigma), and 25 ng/ml amphotericin B (Sigma).

Isolation of human peripheral nerve microvascular endothelial cells forming the BNB

The study protocol for human peripheral nerve tissue was approved by the ethics committee of the Medical Faculty, Yamaguchi University and was conducted in compliance with the Declaration of Helsinki, as amended in Somerset West in 1996. Tissue samples were obtained from an adult female who died of a heart failure. Written informed consent was obtained from the patient's family before the study. PnMECs were isolated in accordance with a previously described procedure (Kanda *et al.*, 1997) with modification. Briefly, the sciatic nerve was removed from the patient, that the epi- and perineuria were carefully stripped off with fine forceps, mimicking the teased fiber preparation for the peripheral nerve pathology. Next, the endoneurium was finely minced with a razor blade and digested with 0.25% collagenase type I (Sigma) in 1×Hanks' balanced salt solution (HBSS) (Invitrogen) at 37°C for 2 hr in a shaking water bath. After centrifugation (800 g, 5 min), the pellet was suspended in 15% dextran solution, followed by centrifugation for 10 min at 4°C and 5,000 g. The pellet was washed and incubated in a type I collagen-coated dish (Iwaki, Chiba, Japan) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Immortalization and purification of PnMECs

After 4 days from the first dissemination, cells were incubated overnight with pDON-AI/tsA58, a retroviral vector encoding the open reading frame of the temperature-sensitive SV40 large T antigen. Twelve hours after the beginning of the incubation, cells were washed with HBSS and were subsequently grown at 33°C. This was followed by another overnight incubation with pDON-AI/hTERT, a retroviral vector encoding the open reading frame of hTERT. After the incubation, cells were washed with HBSS and processed for an additional incubation at 33°C. After endothelial cells (ECs) had proliferated sufficiently for cloning, they were picked up with a cloning cup. As the ECs grew, non-ECs such as pericytes, fibroblasts, and Schwann cells also appeared and gradually began to occupy the culture area of the dish. These non-ECs

were scratched and removed mechanically with a sterilized pointed rubber policeman. After several passages (passage 4) for cloning, some clonal populations that consisted of pure endothelial cells were obtained. We selected one clonal cell line which stably proliferated, and designated these cells as “FH-BNBs”. These FH-BNBs showed immortality at a permissive temperature of 33°C.

Uptake of Dil-Ac-LDL

To label FH-BNBs with 1,1'-dioctadecyl-3,3',3', tetramethyl indocarbocyanine perchlorate acetylated low-density lipoprotein (Dil-Ac-LDL) (Biogenesis, Poole, UK), cells were incubated with 10 µg/ml Dil-Ac-LDL at 33°C in culture medium overnight. The cells were subsequently viewed under a fluorescence microscope (Olympus, Tokyo, Japan). The PnMECs had incorporated bright Dil-Ac-LDL particles into their cytoplasm. Human brain pericyte cell lines which did not take up Dil-Ac-LDL (Shimizu *et al.*, 2011) was used as a negative control.

Cell growth study

To determine the doubling time of the FH-BNBs, 5.0×10^4 cells were seeded on type I collagen-coated 35-mm dishes and grown at 33 and 37°C. After a predetermined period, the cells were trypsinized and counted using a hemocytometer.

Immunocytochemistry

The confluent cultured cells were washed twice in PBS. For von Willebrand factor immunocytochemistry, cells were fixed in 4% paraformaldehyde (Wako, Osaka, Japan) for 15 min at room temperature. Next, cells were permeabilized with 0.1% Triton X-100 (Sigma) for 10 min and then blocked with 1% FBS in PBS for 1 hr. The cells were fixed in 100% methanol for 20 min at -20°C for hTERT immunocytochemistry, were washed three times in PBS, and blocked with 1% FBS. For the staining of claudin-5, ZO-1 and ZO-2, cells were fixed with 100% ethanol for 30 min at 4°C, washed three times in PBS, permeabilized with 1% Triton X-100 for 20 min at room temperature, washed in PBS, and blocked with 2% FBS before staining. For the occludin staining, the cells were fixed with acetone and methanol mixed 1:1 for 10 min at 4°C, washed three times in PBS, and blocked with 2% FBS. After several washes with PBS, cells were incubated with the relevant antibodies (1:50 dilution) at 4°C overnight. Cells were subsequently washed with PBS and then incubated with FITC-conjugated secondary antibodies (1:200 dilutions) for 1 hr at room temperature. HUVECs were used as a negative control for hTERT staining. Human brain pericyte cell lines were used as a negative control (Shimizu *et al.*, 2011). The nuclei were stained with DAPI, and fluorescence was detected using a fluorescence microscope (Olympus).

Transendothelial electrical resistance (TEER) studies

The transwell inserts (pore size, 0.4 µm; effective growth area, 30

mm²; BD Bioscience, NJ, U.S.A.) were coated with rat tail collagen type-I (BD Bioscience) in accordance with the manufacturer's directions. Cells were seeded at 1.0×10^4 cells/insert, on these collagen-coated culture inserts and were incubated at 33°C. After attaching themselves and becoming confluent on the bottom of the insert (24–48 hr), the TEER of cell layers was measured using a Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, FL, U.S.A.). TY08 cells, which are immortalized human brain microvascular endothelial cells that have functional tight junctions (Sano *et al.*, 2010) were used as a positive control. TR-BBBs, derived from the tsA58 transgenic rat brain and lacking claudin-5 expression (Ohtsuki *et al.*, 2007) were used as control endothelial cells without sufficient barrier functions. In addition, we measured the TEER values of FH-BNBs after 3hr incubation with 4 mM EGTA, which disrupt tight junction barrier (Li *et al.*, 2010). Statistical significance was evaluated using Student's *t*-test.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

After reaching confluency, the cells were washed three times with PBS. Total RNA was prepared from PBS-washed cells, human cerebrum and kidney using an RNeasy® Plus Mini kit (Qiagen, Hilden, Germany). RT and PCR amplification were performed with TAKARA PCR Thermal Cycler Dice (Takara, Otsu, Japan). Single-stranded cDNA was synthesized from 250 ng of total RNA using the StrataScript® First Strand Synthesis System (Stratagene, Ceda Creek, TX, U.S.A.) with an oligo-dT primer, and sequential PCR was carried out with Go Taq® (Promega). The temperature cycling conditions for each primer consisted of 5 min at 94°C, 1 min at 55–65°C, and 1 min at 72°C, with a final extension for 10 min at 72°C. The sequence specificity of each primer pair and its reference, except claudin-12, are shown in Table I. The primers for claudin-12 were designed as follows: sense ctgttccataggcagagcg and antisense aagcagattcttagccttc (Accession number NM_012129; fragment size 364 bp). The PCR products were visualized by ethidium bromide staining following resolution on 2% agarose gels. Products were compared with a 50-bp ladder (O'GeneRuler™ 50 bp DNA Ladder, Burlington, Canada) to estimate band size. The size of each amplified product corresponded to the expected size described in the literature.

Permeability studies

FH-BNBs, TY08 cells and TR-BBBs were seeded on 24-well tissue culture inserts (0.4 µm pore size, 1.0×10^4 cells/insert), and grown to confluence. The cells were incubated at 37°C for 30 min before the beginning of the experiment. Next, 1,300 µl of culture medium were added to the lower well and 500 µl of culture medium containing sodium fluorescein (10 µg/ml) and FITC-labeled dextran of molecular weight 4 kDa (1 mg/ml) were added to the upper compartment of each insert. After incubation for 15, 30, 45 or 60 min at 37°C, the lower chamber was sampled and the fluorescence that passed through the cell-covered inserts was mea-

Table I. HUMAN PRIMER PAIRS UTILIZED IN RT-PCR ANALYSIS

molecule	sense	antisense	reference
occludin	5'-ACCCATCTGATATGTGGAA-3'	5'-AGGAACCGCGTGGATTTA-3'	Ghassemifar <i>et al.</i> (2003)
claudin-1	5'-CTGCCCCAGTGGAGGATTTA-3'	5'-CAATGACAGCCATCCACATC-3'	Peng <i>et al.</i> (2011)
claudin-5	5'-CTGTTTCCATAGGCAGAGCG-3'	5'-AAGCAGATTCTTAGCCTTCC-3'	Varley <i>et al.</i> (2006)
claudin-19	5'-CTCAGCGTAGTTGGCATGAA-3'	5'-GAAGAACTCCTGGGTACCA-3'	Peng <i>et al.</i> (2011)
JAM-A	5'-GACACGGGAAGACACTGGGACA-3'	5'-ATGCGCACAGCATTTGAAGTCA-3'	Ghassemifar <i>et al.</i> (2003)
ZO-1	5'-TTTAAGCCAGCCTCTCAACA-3'	5'-CTGCTGGCTTGTTTCTCTAC-3'	Ghassemifar <i>et al.</i> (2003)
ZO-2	5'-GCCTACACTGACAATGAGCTGGA-3'	5'-TTGGGAGCATTATCTTGCTCCTCA-3'	Ghassemifar <i>et al.</i> (2003)
GLUT-1	5'-TGTCCTATCTGAGCATCGTG-3'	5'-CTCCTCGGGTCTCTTATCAC-3'	Umeki <i>et al.</i> (2002)
MDR-1	5'-GCCTGGCAGCTGGAAGACAAATACAAAAATT-3'	5'-CAGACAGCAGCTGACAGTCCAAGAACAGGACT-3'	Weksler <i>et al.</i> (2005)
MRP-1	5'-ACCAAGACGTATCAGGTGGCC-3'	5'-CTGTCTGGGCATCCAGGAT-3'	Weksler <i>et al.</i> (2005)
vWF	5'-CATTGGTGAGGATGGAGTCC-3'	5'-AGCACTGGTCTGCATTCTGG-3'	Fuchs <i>et al.</i> (2006)
G3PDH	5'-TGAAGGTCGGAGTCAACGGATTGGT-3'	5'-CATGTGGGCCATGAGGTCCACCAC-3'	Ye <i>et al.</i> (2003)

sured using a MX3000P instrument (Stratagene). The volume cleared was plotted against time, and the slope estimated by a linear regression analysis was used to calculate the PS value for the endothelial monolayer (PeS), as described previously (Sano *et al.*, 2010): $1/\text{PeS} = 1/P_{\text{total}}S - 1/P_{\text{filter}}S$, where $P_{\text{total}}S$ and $P_{\text{filter}}S$ are the PS values in the presence and absence of cells, respectively. The PeS value was divided by the surface area of the culture insert (30 mm²) to give the endothelial permeability coefficient (Pe).

Western blot (WB) analysis

FH-BNBs and human brain specimens were homogenized in cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% NP40, 0.5% sodium deoxycholate, 150 mM NaCl and a protease inhibitor cocktail tablet (Roche). Homogenized samples were centrifuged at 5,000 g for 10 min at 4°C and the supernatant was subjected to electrophoresis. Twenty micrograms of protein was fractionated on a 7% (p-gp), 10% (β-actin, SV40 T-antigen and GLUT-1) or 12.5% (claudin-5) gel and electrophoretically transferred onto nitrocellulose membranes. The membrane was blocked at room temperature for 2 hrs with 5% powdered skimmed milk in PBS containing 0.05% Tween 20 (PBS-T). After that, the membrane was incubated with a primary antibody in PBS-T and 5% milk (1:100) at room temperature for 2 hrs, followed by incubation with a secondary antibody in PBS-T and 5% milk (1:2000) for 1 hr. Membranes were extensively washed in PBS-T and visualized by enhanced chemiluminescence detection (ECL-plus, Amersham, UK). TY08 cells that express the SV40 large T antigen and claudin-5 were used as a positive control (Sano *et al.*, 2010). HL60 cells, which do not express claudin-5, were used as a negative control, and were purchased from the Japan Health Sciences Foundation (Osaka, Japan). HEK293 cells, which do not express the SV40 large T antigen, were used as a negative control.

Drug accumulation studies

Confluent FH-BNBs grown in type I collagen-coated 24-well plates were washed three times with PBS and preincubated for 30 min at 37°C in a shaking water bath with or without verapamil (100 μM), which is a p-gp efflux transport inhibitor. Next, the cells were incubated in culture medium containing the fluorescent substrates rhodamine 123 (10 μM), a substrate of p-gp and Breast Cancer Resistance Protein (BCRP), or calcein-AM (10 mM), a substrate of p-gp and multi-drug resistance-associated protein 1 (MRP1) with or without verapamil for an hour at 37°C. The cells were then washed three times with PBS and lysed in 1% Triton X-100. The amount of fluorescent substrates retained in the cells was detected using a MX3000P instrument (Stratagene).

Results

Establishment of FH-BNBs

We successfully established a conditionally immortalized peripheral nerve microvascular endothelial cell line, “FH-BNB”, which was obtained by transfection with a retrovirus encoding tsA58 and hTERT. FH-BNBs appeared to be closely packed and have spindle fiber-shaped morphology, which has been thought to characterize endothelial cells constituting barrier systems (Hosoya *et al.*, 2000) (Fig. 1A). Endothelial origin with FH-BNB was confirmed by their expression of von Willebrand factor and the uptake of Dil-Ac-LDL, both of which have been reported to be specific markers for endothelial cells (Yamamoto *et al.*, 1998; Voyta *et al.*, 1984). All of these cells were positive for Dil-Ac-LDL incorporation (Fig. 1A and B). The expression of factor VIII/von Willebrand factor, a specific marker for endothelial cells, on these cells was detected by RT-PCR (Fig. 1C) and immunocytochemical analysis (Fig. 1D).

Fig. 1. A phase contrast micrograph of the FH-BNBs (A) and the corresponding fluorescence image after Dil-Ac-LDL uptake (B). The mRNA expression of von Willebrand factor (C) in FH-BNBs determined by RT-PCR analysis. The reactions were performed against total RNA with (+) or without (–) the RT reaction. Immunostaining of FH-BNBs (D) using an anti-von Willebrand factor antibody. Human brain pericyte cells did not take up Dil-Ac-LDL (E) and express von Willebrand factor (F). The scale bars correspond to 100 μ m.

Expression of *tsA 58* and *hTERT*

In FH-BNBs, hTERT proteins were found in their nuclei (Fig. 2A and B). In addition, a WB analysis showed that these cells exhibited the large T-antigen with a molecular weight of 94 kDa, which was the same molecular weight as that of the TY08 cells used as a positive control (Fig. 2D). After the temperature shift from 33°C to 37°C, the amount of large T-antigen gradually decreased (Fig. 2D). The amount of large-T antigen protein decreased dramatically 48 hrs after the temperature shift. When FH-BNBs were seeded and cultured at 33°C, they proliferated stably with a doubling time of about 3 days (Fig. 2E) and maintained their spindle shaped morphology for more than 30 passages. However, two days after the temperature shift from 33 to 37°C, the cell growth arrested and the cells gradually swelled and finally died after a ~7-day culture (Fig. 2F). These results indicated that FH-BNBs have an obvious temperature sensitive nature and are dissimilar to cell lines expressing both the SV40 T antigen and hTERT.

Expression of tight junction molecules in FH-BNBs

The expression of claudin-1, claudin-5, claudin-12, junctional adhesion molecule A (JAM-A), ZO-1 and ZO-2 corresponding to tight junction components of endothelial cells forming BBB (Zlokovic, 2008) was verified in FH-BNBs by an RT-PCR analysis (Fig. 3A, B). Claudin-19, which deficient mice induced peripheral nervous system deficits (Miyamoto *et al.*, 2005) was expressed in FH-BNBs, but not in human cerebrum. Claudin-5, the most important barrier protein, was detected by a WB analysis at a molecular weight of 25 kDa (Fig. 3C). In addition, we showed that claudin-5, occludin, ZO-1 and ZO-2 were localized at the cell-cell boundaries in FH-BNBs by an immunohistochemical analysis (Fig. 4A–D).

Expression of transporters and functional expression of *p-glycoprotein* in FH-BNBs

The mRNA expression of GLUT-1, MDR1a and MRP1 were detected in FH-BNBs (Fig. 5A). In addition, GLUT-1 with a molecular weight of 55 kDa, and p-gp encoded by

Fig. 2. A phase microscopy image of FH-BNBs (A) and the corresponding immunocytochemical staining using an anti-hTERT antibody (B). Both nuclear (arrow) and perinuclear (arrowhead) hTERT immunoreactivities were detected. HUVECs were used as a negative control for hTERT staining (C). The scale bars indicate 25 μ m. The quantity of tsA58 protein (D) in FH-BNBs at permissive or non-permissive temperature was examined using an anti-SV40 large T antigen antibody. Lane 1, TY08 cells used as a positive control; Lane 2, FH-BNBs cultured at 33°C; Lane 3, FH-BNBs cultured for 24 hrs at 37°C; Lane 4, FH-BNBs cultured for 48 hrs at 37°C; Lane 5, FH-BNBs cultured for 3 days at 37°C; Lane 6, FH-BNBs cultured for 5 days at 37°C. The FH-BNBs proliferated readily at the permissive temperature of 33°C; Lane 7, HEK293 cells used as a negative control. (E). About 2 days after the temperature shift to 37°C, the cell growth was arrested. Each point represents the mean $\pm$ standard deviation (SD, $n=3$). The temperature sensitive nature of FH-BNBs (F). This cell line demonstrated sufficient proliferation at the permissive temperature (33°C). However, once the cells are spread at the dish at 37°C, they gradually lose their proliferative capacity and become senescent and enlarged. This morphology resembles non-transfected, primary cultured human PnMECs.

Fig. 3. The RT-PCR analysis of the expression of tight junction molecules in FH-BNBs. The expression of claudin-1, claudin-5, claudin-12, JAM-A, ZO-1 and ZO-2 mRNA in human brain specimens (lane 1) and FH-BNBs (lane 2) were verified (A). Claudin-19 mRNA were expressed in human kidney specimens (lane 1) and FH-BNBs (lane 3) but not human cerebrum specimens (lane 1) (B). G3PDH was used as a control. The reactions were performed against total RNA with (+) or without (-) RT. The Western blot analysis of claudin-5 in FH-BNBs (C). Lane 1, TY08 cells used as a positive control; Lane 2, HL-60 cells as a negative control; Lane 3, FH-BNBs.

MDR-1 with a molecular weight of 190 kDa, were verified in FH-BNBs at the protein level by a WB analysis (Fig. 5B). Next, the function of p-gp was evaluated in the FH-BNBs. The influence of the p-gp inhibitor verapamil on the uptake of the p-gp substrates rhodamine 123 and calcein-AM in the FH-BNBs was investigated. The inhibition of p-gp by verapamil led to a significantly increased uptake of rhodamine123 (Fig. 6A) and calcein-AM (Fig. 6B). These results therefore showed that the p-gp expressed in the FH-BNBs acted as an efflux pump against the p-gp substrates.

FH-BNBs have functional tight junctions

Compared with TY08 cells, which express claudin-5 and have functional tight junctions (Sano *et al.*, 2010), FH-BNBs demonstrated the similar degrees of the permeability coefficients for sodium fluorescein (MW=376 Da; 0.60 ± 0.16 vs $0.34 \pm 0.03 \times 10^{-3}$ cm/min) (Fig. 7A) and FITC-dextran (MW=4 kDa; 0.34 ± 0.12 vs $0.30 \pm 0.09 \times 10^{-3}$ cm/min) (Fig. 7B), as well as similar TEER values (36.3 ± 4.0 vs $38.0 \pm 4.4 \Omega \cdot \text{cm}^2$) (Fig. 7C). In addition, the permeabilities to each of the above compounds were much lower than those of TR-BBBs and the TEER value of FH-BNBs was much higher than that of the TR-BBBs, which have been reported to lose their barrier properties due to a lack of claudin-5

expression (Ohtsuki *et al.*, 2007). The TEER values of FH-BNBs incubated with 4 mM EGTA, which disrupt tight junction barrier (Li *et al.*, 2010), decreased and became comparable to those of TR-BBBs (Fig. 7C). These results indicate that FH-BNBs have the same degree of functional tight junctions as TY08 cells.

Discussion

In this study, we successfully established a human peripheral nerve microvascular endothelial cell line (FH-BNBs). Since primary PnMECs rapidly undergo senescence after a few divisions (Kanda *et al.*, 1997), it is very difficult to establish stable experimental systems using primary cell culture. Usually, oncogenes such as the SV40 large T-antigen, have been used to immortalize human somatic cells like brain microvascular endothelial cells (Stins *et al.*, 2001). However, the SV40 large T-antigen interacts with various proteins such as retinoblastoma and p53, and these interactions often affect fundamental cellular functions. In fact, one of the endothelial cell lines immortalized by the SV40 large T-antigen did not show contact inhibition, i.e. they demonstrated a tumor-like phenotype (Callahan *et al.*, 2004). To overcome these problems, we have immortalized

Fig. 4. Immunocytochemical analysis of tight junction proteins in confluent FH-BNBs. Claudin-5 (A), occludin (B), ZO-1 (C) and ZO-2 (D) were continuously detected at the cell-cell boundaries in FH-BNBs. Their nuclei were stained with DAPI (blue). The scale bars indicate 25 μm.

Fig. 5. (A) The expression of transporters in FH-BNBs. G3PDH was used as a control. MDR-1, MRP-1 and GLUT-1 mRNA were all detected in human brain samples as well as in FH-BNBs. The reaction were performed against total RNA with (+) or without (-) RT. (B) The Western blot analysis of the expression of p-gp and GLUT-1 in FH-BNBs. Lane 1, human cerebral cortex as a positive control; Lane 2, FH-BNBs.

Fig. 6. The result of the p-gp functional assay in FH-BNBs. The cellular uptake of rhodamine123 (A) and calceinAM (B), which are substrates of p-gp was measured in the presence or absence of verapamil, a specific inhibitor of p-gp. The uptake of rhodamine123 and calceinAM in the presence of verapamil was significantly higher than that of control cells without verapamil. The experiments were performed in triplicate. The data are presented as the means and SD.

Fig. 7. The paracellular transport of sodium fluorescein (A) and FITC-dextran (B) in FH-BNBs, TY08 cells and the TR-BBBs. The permeability coefficients (Pe) of FH-BNBs for these compounds were as low as those of TY08 cells, and were much lower than those of the TR-BBBs. (C) The transendothelial electrical resistance (TEER) values of the FH-BNBs were as high as those of the TY08 cells, and were higher than those of the TR-BBBs. The TEER values of FH-BNBs incubated with 4 mM EGTA decreased and became comparable to those of TR-BBBs. The experiments were performed in triplicate. The data are presented as the means $\pm$ SD.

primary human PnMECs a using temperature-sensitive SV 40 large T antigen (tsA58) and the catalytic subunit of human telomerase (hTERT). FH-BNBs, which express both the hTERT protein and the tsA58 protein, showed a temperature-sensitive nature (Fig. 2E). They grow stably

under the permissive temperature of 33°C and stop growing under the non-permissive temperature of 37°C. FH-BNBs showed spindle fiber morphology and contact inhibition even under the permissive temperature of 33°C, indicating that FH-BNBs, even at the permissive temperature, did not

develop a tumor-like phenotype.

The endothelial cells forming the BBB express many TJ-associated molecules including occludin (Furuse *et al.*, 1993), claudin-1 (Pfeiffer *et al.*, 2011), claudin-5 (Nitta *et al.*, 2003), claudin-12 (Nitta *et al.*, 2003; Ohtsuki *et al.*, 2007), ZO-1, ZO-2 (Biernacki *et al.*, 2004) and JAM-A (Yeung *et al.*, 2008). These molecules compose TJs and limit the paracellular permeability in order to maintain the brain microenvironment. Previous reports have indicated that PnMECs in human sural nerves express occludin, claudin-5 and ZO-1 (Kanda *et al.*, 2004), and claudin-1 was detected in perineurium (Hirakawa *et al.*, 2003), primary human PnMECs (Yosef *et al.*, 2010) and human Schwann cells (Alanne *et al.*, 2009). We also reported that rat PnMECs expressed many molecules forming tight junctions (Sano *et al.*, 2007). FH-BNBs expressed all of the important molecules for maintaining tight junctions at the mRNA level, and contained occludin, claudin-5, ZO-1 and ZO-2 at the cell-cell boundaries at the protein level. Claudin-19 was expressed in human retinal pigment epithelium (Peng *et al.*, 2011) and mouse sciatic nerve and kidney, but not in brain (Miyamoto *et al.*, 2005). Furthermore, claudin-19 existed in peripheral myelinated axons and claudin-19 deficient mice induced peripheral nervous system deficits (Miyamoto *et al.*, 2005). We firstly demonstrated that human PnMECs express claudin-19, indicating that claudin-19 might be a factor to differentiate the endothelial cells of BNB from those of BBB. Trans endothelial permeability studies in FH-BNBs demonstrated that these cells have a barrier function similar to TY08 cells. Furthermore, the TEER values and permeability to FITC-labeled dextran (MW=4 kDa) were similar to those of hCMEC/D3 cells (TEER 36.3 ± 4.0 vs $30-40 \Omega \cdot \text{cm}^2$; P_e 0.34 ± 0.12 vs $0.325 \times 10^{-3} \text{ cm/min}$), which are human brain microvascular endothelial cell lines that maintain functional tight junctions (Weksler *et al.*, 2005). In the case of sodium fluorescein, the permeability of the FH-BNBs was lower than that of the hCMEC/D3 cells ($0.60 \pm 0.16 \times 10^{-3} \text{ cm/min}$ vs $2.36 \times 10^{-4} \text{ cm/sec}$) (Hsueh *et al.*, 2010). Consequently, the FH-BNBs were thought to form an effective barrier similar to the BBB forming endothelial cell lines, TY08 and hCMEC/D3 cells. It has been reported that immortalized endothelial cell lines derived from mouse, bovine, rat, porcine BBB show high TEER values (Deli *et al.*, 2005). On the other hand, the TEER of hCMEC/D3 cells and TY08 cells, both of which were derived from human brain, have been reported to be $30-40 \text{ ohm cm}^2$ (Weksler *et al.*, 2005; Sano *et al.*, 2010). We think that this discrepancy of the TEER values might result from a species differences. Another considerable point is the problem of purity of the cells. Yosef *et al.* reported that primary human PnMECs have higher TEER values than FH-BNBs (Yosef *et al.*, 2010). However, these primary cells inevitably contained a small amount of pericytes, which have been reported to promote barrier integrity of the barrier-derived endothelial cells (Shimizu *et al.*, 2011).

Because the purity of FH-BNBs, TY08, and hCMEC/D3 is 100% and all of these cell lines are derived from humans, we think that FH-BNBs have functional tight junctions as barrier-constituting cells derived from human tissue.

In the brain, ABC transporters such as p-gp and MRP-1 are mainly localized on the luminal surface of endothelial cells, and quickly remove the toxic metabolites to maintain brain homeostasis (Schinkel *et al.*, 1996). GLUT-1, a D-glucose transporter, is abundantly present on the abluminal surface of endothelial cells forming the BBB. GLUT-1 plays important roles in brain metabolism, since glucose is the main energy source in the brain (Qutub and Hunt, 2005; Simpson *et al.*, 2007). The glucose requirements of peripheral nerves have been also reported to be very high (Greene and Winegrad, 1979). We previously described that the same transporters were expressed in rat PnMECs (Sano *et al.*, 2007). GLUT-1 and p-gp have been demonstrated to present on microvessels in the endoneurium of rat sciatic nerves by an immunohistochemical analysis (Sano *et al.*, 2007). The FH-BNBs in the present study also expressed MDR-1, MRP-1 and GLUT-1. In addition, the functional expression of p-gp was observed in these cells. These results suggest that PnMECs supply D-glucose as an energy source for nerve fibers by using GLUT-1, and protect nerve tissues against toxic substrates with p-gp in the peripheral nervous system.

Recently, primary cultured human PnMECs were isolated and extensively characterized (Yosef *et al.*, 2010). These cells are very useful to analyze the nature of the endothelium forming the BNB; however, they have limited capacities for division because they are not immortalized, and they also contain a small percentage of non-endothelial cells including pericytes and fibroblasts. On the other hand, our FH-BNBs consist of 100% endothelial cells derived from the BNB and have the ability to grow and maintain their barrier phenotype after at least 30 passages.

In conclusion, the newly established FH-BNBs, which maintain their original nature as endothelial cells forming the BNB will be a useful tool for investigating the biology of the human BNB, and may lead to the development of novel therapies for many autoimmune and other neuropathies.

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