Endothelial Cells Constituting Blood-nerve Barrier Have Highly Specialized Characteristics as Barrier-forming Cells

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ABSTRACT. In autoimmune disorders of the peripheral nervous system (PNS) such as Guillain-Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy, breakdown of the blood-nerve barrier (BNB) has been considered as a key step in the disease process. Hence, it is important to know the cellular property of peripheral nerve microvascular endothelial cells (PnMECs) constituting the bulk of BNB. Although many *in vitro* models of the blood-brain barrier (BBB) have been established, very few *in vitro* BNB models have been reported so far. We isolated PnMECs from transgenic rats harboring the temperature-sensitive SV40 large T-antigen gene (tsA58 rat) and investigated the properties of these “barrier-forming cells”. Isolated PnMECs (TR-BNBs) showed high transendothelial electrical resistance and expressed tight junction components and various types of influx as well as efflux transporters that have been reported to function at BBB. Furthermore, we confirmed the *in vivo* expression of various BBB-forming endothelial cell markers in the endoneurium of a rat sciatic nerve. These results suggest that PnMECs constituting the bulk of BNB have a highly specialized characteristic resembling the endothelial cells forming BBB.

Key words: blood-nerve barrier/peripheral nerve microvascular endothelial cells/tight junction/claudin-5/transporter

Introduction

The blood-nerve barrier (BNB) is one of the functional barriers sheltering the nervous system from circulating blood (Poduslo *et al.*, 1994). BNB comprises the endoneurial microvasculature and the innermost layers of the perineurium. Tight junctions (TJs) between adjacent peripheral nerve

microvascular endothelial cells (PnMECs) and between perineurial cells, in addition to the lack of vesicular transport, are responsible for BNB function (Bell *et al.*, 1984; Latker *et al.*, 1991). Blood-borne substances can reach the endoneurial extracellular space by crossing either the endoneurial vascular endothelium or the perineurium. Many studies (Soderfeldt, 1974; Boddingius, 1984; Weerasuriya and Rapoport, 1986; Weerasuriya *et al.*, 1989) indicated that perineurial permeability is much lower than that of endoneurial microvessels against various substances. Hence, PnMECs constituting the bulk of microvessels in the endoneurium can be considered the real interface between blood and peripheral nerves. Recently, breakdown of BNB has been considered as an initial key step in many autoimmune disorders of the peripheral nervous system including Guillain-Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), and paraprotemic neuropathy (Lach *et al.*, 1993; Kanda *et al.*, 1994, 2000,

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Abbreviations: BSA, bovine serum albumin; SV40, simian virus 40; PBS, phosphate-buffered saline; EC, endothelial cell; CNS, central nervous system; PNS, peripheral nervous system; tsA58 rat, transgenic rat harboring temperature-sensitive SV40 large T-antigen gene; TR-BNB, conditionally immortalized rat brain capillary endothelial cell line; TR-BNB, conditionally immortalized rat peripheral nerve microvascular endothelial cell line.

2004). Because PnMECs are the structural basis of BNB, as indicated above, investigation of the cellular characteristics of PnMECs using cell culture technique may provide new insights into the pathogenesis of immune-mediated neuropathies. To date, brain microvascular endothelial cells (BMECs) have been successfully isolated in many laboratories and utilized for studying the molecular and cellular basis of BBB (Terasaki *et al.*, 2003). On the other hand, only a few reports concerning the successful culture of PnMECs have been available thus far (Argall *et al.*, 1994; Kanda *et al.*, 1997; Iwasaki *et al.*, 1999). This is probably due to the technical difficulty in isolating PnMECs from a peripheral nerve. Although many endothelial cells isolated from humans and animals need to be introduced immortalizing genes, such as the SV 40 large T-antigen, to gain abilities for stable proliferations, these immortalized cells often end up losing their natural properties *in vivo* (Terasaki *et al.*, 2003). Recently, transgenic rats harboring temperature-sensitive simian virus 40 large T-antigen (tsA58) gene have been developed as a source of conditionally immortalized cell lines (Takahashi *et al.*, 1999). Cultured cells derived from this animal can be easily immortalized by activating the tsA58 gene at a permissive temperature of 33°C and can have *in vivo* functions at a nonpermissive temperature of 37°C or 39°C (Takahashi *et al.*, 1999; Hosoya *et al.*, 2000). Here, we successfully isolated PnMECs from the tsA58 rats and investigated the properties of these cells. In addition, we examined whether *in vivo* microvessels in the endoneurium express barrier-related molecules, which have been reported to function at BBB. This study should establish a new paradigm for understanding the nature of PnMECs forming the bulk of BNB.

Materials and Methods

Animals

The origin and characteristics of the transgenic rats harboring the temperature sensitive SV40 large T antigen gene have been previously described (Takahashi *et al.*, 1999). Wistar rats utilized for immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) analysis were purchased from Charles River Laboratories Inc. (Yokohama, Japan). All investigations using rats described in this report conform to the requirements established by the Institute of Laboratory Animals, Yamaguchi University Graduate School of Medicine.

Culture media

Dissecting medium (DM) contained Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, U.S.A.) supplemented with 5% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin (Sigma), 100 µl/ml streptomycin (Sigma), and 25 ng/ml amphotericin B (Invitrogen, Grand Island, NY, U.S.A.). Endothelial cell

(EC) growth medium contained EBM-2 medium (Cambrex, Walkersville, MD, U.S.A.) supplemented with 20% FBS, EGM-2 MV (Cambrex), 100 U/ml penicillin (Sigma), 100 µl/ml streptomycin (Sigma), and 25 ng/ml amphotericin B (Invitrogen).

Isolation of tsA58 rat PnMECs forming BNB and endothelium from tsA58 rat aorta

PnMECs were isolated in accordance with a previously described procedure (Kanda *et al.*, 1997) with modification. Briefly, the sciatic nerve was removed from a rat killed by euthanasia procedures. 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×Hank's balanced salt solution (HBSS) (Invitrogen) at 37°C for 2 hr. After centrifugation, the pellet was washed and placed onto a type I collagen-coated dish. Cells were cultured at 37°C during the first 48–72 hr to allow the cells to attach themselves to the dish. Cells were subsequently cultured at 33°C in a humidified atmosphere of 5% CO₂ and 95% air. When the EC colonies grew sufficiently for cloning, they were picked up with a cloning cup. As 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. After that, ECs were isolated and cultured for 2–3 weeks at 33°C. These PnMECs (TR-BNBs) showed immortality at a permissive temperature of 33°C. ECs from tsA58 rat aorta were isolated in accordance with a previously reported method (Kobayashi *et al.*, 2005).

Identification of PnMECs

PnMECs were identified by the following three criteria: spindle-fiber-shaped morphology; immunoreactivity to an anti-von Willebrand factor antigen antibody, and uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate acetylated low-density lipoprotein (DiI-Ac-LDL) (Biogenesis, Poole, England). To label with DiI-Ac-LDL, cells were incubated with 10 µg/ml DiI-Ac-LDL at 33°C in a culture medium overnight. Cells were subsequently viewed under a fluorescence microscope (Olympus, Tokyo, Japan). PnMECs incorporated bright DiI-Ac-LDL particles into their cytoplasm.

Immunocytochemistry

Cells (1×10⁶) were cultured on a rat tail collagen type I-coated 30-mm dish (Becton Dickinson) at 33°C until reaching confluence (24–48 hr). After an additional incubation at a nonpermissive temperature of 37°C for 24 hr, cells were washed three times with 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% BSA in PBS for 1 hr. After several washes with PBS, cells were incubated

with the mouse anti-human von Willbrand factor antibody (1:100 dilution) (Dako, A/S, Denmark) with blocking solution at 4°C overnight. Cells were subsequently washed with PBS and then incubated with FITC-conjugated anti-mouse IgG (1:100 dilution) (Zymed, CA, U.S.A.) for 1 hr at room temperature. For claudin-5 immunocytochemistry, cells were fixed in 100% ethanol for 30 min at 4°C. After treatment with 1% Triton X-100/PBS for 10 min at room temperature, the cells were blocked with 1% BSA/PBS for 1 hr. After several washes with PBS, cells were incubated with a rabbit anti-mouse claudin-5 antibody (1:100 dilution) (Zymed, CA, U.S.A.) with a blocking solution at 4°C overnight. Cells were subsequently washed with PBS and then incubated with FITC-conjugated anti-rabbit IgG (1:100 dilution) (Zymed, CA, U.S.A.) for 1 hr at room temperature. Fluorescence was detected with a fluorescence microscope (Olympus).

Transendothelial electrical resistance (TEER) study

Transendothelial electrical resistance (TEER) reflects the barrier properties of the endothelium (Deli *et al.*, 2005). The transwell inserts (pore size, 0.4 μ m; effective growth area, 0.3 cm²; BD Bioscience, New Jersey, U.S.A.) were coated with rat tail collagen type-I (BD Bioscience) in accordance with the manufacturer's instruction. At 1.0×10^6 cells/insert, cells were seeded on these collagen-coated culture inserts at the nonpermissive temperature (37°C) (Hosoya *et al.* 2000). After attaching themselves with confluence to the bottom of the insert (24–36 hr), the TEER of cell layers was measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, FL, U.S.A.). TR-BBB13 cells, derived from the tsA58 rat brain, which have been thought to suppress claudin-5 expression (Hosoya *et al.*, 2000; Ohtsuki *et al.*, 2007) and human umbilical vein endothelial cells (HUVECs) (Japan Health Sciences Foundation, Osaka, Japan) were used as control endothelial cells without barrier properties. Statistical significance was evaluated using Student's t-test.

RT-PCR analysis

After an incubation at the nonpermissive temperature of 37°C for 24 hr, cells were washed three times with PBS. Total RNA was prepared from PBS-washed cells or the rat cerebrum using an RNeasy® Plus Mini kit (Qiagen, Hilden, Germany). RT and PCR amplification were carried out with TAKARA PCR Thermal Cycler Dice (TakaRa, Otsu, Japan). Single-stranded cDNA was synthesized from 50 ng of total RNA using the StrataScript® First Strand Synthesis System (STRATAGENE®, Cedar Creek, TX, U.S.A.) with an oligo-dT primer, and sequential PCR was performed with TaKaRa Ex Taq® (TaKaRa). Temperature cycling conditions for each primer consisted of 5 min at 94°C followed by 35–40 cycles for 1 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 rat primer pair and its reference are shown in Table I. The PCR products were visualized by ethidium bromide staining following resolution on 2% agarose gel. Products were

compared with a 50-bp ladder (Fermentas, Burlington, Canada) to estimate band size. The size of each amplified product corresponded to the expected size described in each literature we had referred. For the RT-PCR analysis of rat tissues, the cerebrum, sciatic nerve, aorta, and kidneys were removed from a nontransgenic Wistar rat killed by euthanasia procedures. The cerebral cortex, endoneurium of a sciatic nerve, aorta, and kidneys were separately minced and homogenized for total RNA extraction. After single-stranded cDNA was synthesized from 400 ng of total RNA derived from each tissue, PCR and sequential examinations were performed using the same procedure described above.

Immunohistochemistry

Rabbit polyclonal antibodies against human claudin-12, human occludin and mouse claudin-5 were purchased from Zymed (San Francisco, CA, U.S.A.). A rabbit polyclonal antibody against human GLUT-1 was purchased from Santa Cruz Biotechnology (CA, U.S.A.). A mouse monoclonal antibody against human p-glycoprotein (p-gp) was purchased from Signet Laboratories (Dedham, MA, U.S.A.). The brain and sciatic nerve were removed from a Wistar rat killed by the euthanasia procedures. Tissues were snap-frozen by immersion into isopentane/liquid nitrogen. Cryostat sections (10 μ m) were mounted on poly-L-lysine-coated slides and air-dried. After a 15-min fixation with 4% paraformaldehyde at 4°C, they were exposed to 0.3% H₂O₂/methanol for 10 min at room temperature. Sections were then preincubated in PBS supplemented with 10% normal goat serum for 1 hr before incubating overnight with the primary antibody diluted in PBS at 4°C. The anti-occludin antibody was used at 1/800 dilution, whereas others were used at 1/400 dilution. Sections were then rinsed with PBS three times before incubation for 1 hr with peroxidase-conjugated secondary antibodies (Nichirei, Tokyo, Japan) at room temperature. The reaction product indicating immunoreactivity in sections was developed with diaminobenzidine (Nichirei).

Results

Establishment of TR-BNBs

We had successfully isolated PnMECs from sciatic nerves of a tsA58 rat. TR-BNBs were closely packed and showed a spindle-fiber-shaped morphology that has been well recognized to be ECs constituting the barrier system (Kanda *et al.*, 1997; Hosoya *et al.*, 2000) (Fig. 1A). In contrast, ECs of the tsA58 rat aorta, as representative cells that do not form the blood-tissue barrier, showed a “cobblestone-like appearance”, not a spindle-fiber-shaped morphology (Fig. 1B). Almost 100% of the cells were positive for DiI-Ac-LDL, indicating excellent purity (Fig. 1C, D). The endothelial origin of these cells was also supported by the detections of the factor VIII/vWF antigen by RT-PCR analysis (Fig. 1E) and immunocytochemistry (Fig. 1F).

Fig. 1. Spindle-fiber-shaped morphology and endothelial properties of isolated TR-BNBs. (A): Phase contrast micrograph of TR-BNBs cultured at 33°C. (B): Cobblestone-like appearance of tsA58 rat aortic endothelial cells. (C) and (D): Fluorescence micrograph of TR-BNBs with DiI-Ac-LDL (C) and phase contrast micrograph of the same field (D). (E): Expression of von Willebrand factor gene in TR-BNBs detected by RT-PCR analysis. F: Immunostaining of TR-BNBs using anti-von Willebrand factor antibody. Scale bars, 100 μ m.

TR-BNBs expressing claudin-5 have high TEER

The TEER of TR-BNBs was significantly higher than that of HUVECs (Fig. 2A). Furthermore, the TEER of TR-BNBs was also higher than that of TR-BBBs (TR-BBB13) (Fig. 2A), which have been reported to suppress claudin-5 expression (Ohtsuki *et al.*, 2007). In addition, we investigated whether the TR-BNBs express claudin-5. Claudin-5 was localized at the cell-cell boundaries in TR-BNBs (Fig. 2B).

Expression of tight junction molecules in TR-BNBs

To investigate the mRNA expression of components of tight junctions in PnMECs, total RNA was isolated from TR-BNBs and processed for RT-PCR analysis. The expressions of occludin, claudin-5, claudin-12, junctional adhesion molecule 1 (JAM1), zonula occludens 1 (ZO-1), and zonula occludens 2 (ZO-2), corresponding to tight junction components of ECs forming BBB (Lai *et al.*, 2005) were confirmed in TR-BNBs (Fig. 3). The sizes of amplified

fragments of each molecule were identical to previously reported values (Table I).

Expression of influx transporters in TR-BNBs

The mRNA expression of various blood-to-brain influx transporters in TR-BNBs was examined by RT-PCR analysis using a specific primer set for each rat transporter (Table I). Glucose transporter 1 (GLUT1), which takes in D-glucose from circulating blood, was expressed in TR-BNBs (Fig. 4). The expression of a large amino acid transporter (system L), which consists of large neutral amino acid transporter-1 (LAT-1) and 4F2hc, was examined. LAT-1 and 4F2hc mRNAs were amplified at their expected sizes (Fig. 4). Creatine plays a pivotal role in the storage of phosphate-bound energy in the brain, and creatine transporter (CRT) supplies creatine to the brain via BBB. (Ohtsuki *et al.*, 2002). CRT was also expressed in TR-BNBs (Fig. 4). On the other hand, monocarboxylic acid transporter 1 (MCT1), which has been reported to work as a blood-to-brain transporter of monocarboxylic acids such as lactate (Kido *et al.*,

Fig. 2. TEER and claudin-5 expression in TR-BNBs. (A) Transendothelial electrical resistance (TEER) values of TR-BNBs, TR-BBBs, and HUVECs. TEER values recorded in ohms (Ω) $\times$ insert area were corrected to Ωcm^2 . TEER values of inserts with medium alone were subtracted from TEER values of inserts with confluent monolayers of each cell line. (mean $\pm$ SD, n=3 in each experiment). (B) Expression and localization of claudin-5 in TR-BNBs. Claudin-5 was detected at the cell-cell boundaries of TR-BNBs. Scale bars, 100 μm .

Fig. 3. mRNA expression of TJ molecules in TR-BNBs. Expression of occludin, claudin-5, claudin-12, ZO-1, ZO-2, and JAM1 mRNAs in the rat cerebrum and TR-BNBs was investigated by RT-PCR analysis with a specific primer set for each gene. Reactions were performed against total RNA with (+) or without (-) reverse transcription. The amplified products for all molecules were detected in TR-BNBs.

Fig. 4. mRNA expression of influx transporters in TR-BNBs. Expression of GLUT1, LAT-1, 4F2hc, MCT1, and CRT mRNAs in the rat cerebrum and TR-BNBs was investigated by RT-PCR analysis with a specific primer set for each gene. Reactions were performed against total RNA with (+) or without (-) reverse transcription. The amplified products for all molecules but MCT1 were detected in TR-BNBs.

2000), was not expressed in TR-BNBs (Fig. 4).

Expression of brain-to-blood efflux transporters in TR-BNBs

To elucidate whether PnMECs at BNB, similarly to BMECs at BBB, carry out active efflux transport of various drugs

and unnecessary metabolites for neurons from the peripheral nervous parenchyma to the inner lumen of a capillary, we investigated mRNA expression of diverse brain-to-blood efflux transporters that have been reported to function at BBB. Multidrug resistance gene 1a (Mdr1a), multidrug resistance associated protein 1 (Mrp1), and ATP-binding cassette subfamily G member 2 (ABCG2), which mediate

Table I. RAT PRIMER PAIRS UTILIZED IN RT-PCR ANALYSES

molecule	sense	antisense	reference
Occludin	5'-GCCTTTTGCTTCATCGCTTCC-3'	5'-AACAAGATTAAAGCAAAAGCCAC-3'	Hosoya <i>et al.</i> , 2000
Claudin-5	5'-GCAGAGTACAGGGCACATGC-3'	5'-TAGTTCTTCTTGTCGTAATCGCC-3'	Hosoya <i>et al.</i> , 2000
Claudin-12	5'-GCGGCCGCGACAGGCTGTTTGGAGAA-3'	5'-GCGGCCGCCACTGATGGGTAAGGAACA-3'	Ohtsuki <i>et al.</i> , 2007
JAM1	5'-ACAGCCATGAGGTCAGAGGCT-3'	5'-ACCTAGAAGACATTGAAGGCATC-3'	Tomi and Hosoya, 2004
ZO-1	5'-GCGAGGCATCGTTCCTAATAAG-3'	5'-TCGCCACCTGCTGTCTTTG-3'	Shi and Zheng, 2005
ZO-2	5'-TCACAGCTGCAGGTGTCACA-3'	5'-TGCTGTTCTGGGTTGAGGAAC-3'	Shi and Zheng, 2005
GLUT-1	5'-GATGATGAACCTGTTGGCCT-3'	5'-AGCGGAACAGCTCCAAGATG-3'	Tomi and Hosoya, 2004
4F2hc	5'-CTCCCAGGAAGATTTTAAAGACCTTCT-3'	5'-TTCATTTTGGTGGCTACAATGTCAG-3'	Hosoya <i>et al.</i> , 2000
LAT-1	5'-CAAGCTCTGGATCGAGCTGCTC-3'	5'-TGTGGGTGGATCATGGAGAGG-3'	Hosoya <i>et al.</i> , 2000
MCT1	5'-GAAAAACTCAAGTCCAAAGAGTCT-3'	5'-AGAAGCCCCAAGAAGACAATGAAA-3'	Hosoya <i>et al.</i> , 2001
CRT	5'-GAAATGGTGTGCTGCTTCTTTCAC-3'	5'-GTCACATGACACTCTCCACCACGA-3'	Tomi and Hosoya, 2004
Mdr1a	5'-ACAGAAACAGAGGATCGC-3'	5'-CGTCTTGATCATGTGGCC-3'	Hosoya <i>et al.</i> , 2000
Mrp1	5'-CTGGCTTGGTGTGAAGTAT-3'	5'-AGGCTCTGGCTTGGCTCTAT-3'	Hosoya <i>et al.</i> , 2000
ABCG2	5'-CAATGGGATCATGAAACCTG-3'	5'-GAGGCTGATGAATGGAGAA-3'	Hosoya <i>et al.</i> , 2004
OAT3	5'-ATCTCATCAACATCTATTGGCT-3'	5'-CAGAGAGAGACAGAAGGTCA-3'	Ohtsuki <i>et al.</i> , 2005
Oatp2	5'-GAGTACCTTCTGTCTTTCCTTAGCTA-3'	5'-AACTAACGCAATCTGGCTTAACCAAG-3'	Ito <i>et al.</i> , 2003
vWF	5'-GCCTCTACCAGTGAGGTTTGAAG-3'	5'-ATCTCATCTCTTCTTCTGCTCCAGC-3'	Hosoya <i>et al.</i> , 2000
GAPDH	5'-TGATGACATCAAGAAGGTGGTGAAG-3'	5'-TCCTTGGAGGCCATGTAGGCCAT-3'	Ohtsuki <i>et al.</i> , 2005

Fig. 5. mRNA expression of brain-to-blood efflux transporters in TR-BNBs. Expression of Mdr1a, Mrp1, ABCG2, OAT3, and oatp2 mRNAs in the rat cerebrum and TR-BNBs was investigated by RT-PCR analysis with a specific primer set for each gene. Reactions were performed against total RNA with (+) or without (–) reverse transcription. Although the amplified products for Mdr1a, Mrp1, and ABCG2 were detected in TR-BNBs, those for OAT3 and oatp2 were not detected.

brain-to-blood efflux transport at BBB, were all detected at the mRNA level in TR-BNBs (Fig. 5). However, organic anion transporter 3 (OAT3) and oatp2, which are other types of brain-to-blood efflux transporter, were not detected

Fig. 6. mRNA expression of barrier-related molecules in various organs from a nontransgenic Wistar rat. Expression of Mdr1a, GLUT-1, occludin, claudin-5, and claudin-12 in rat tissues was investigated with primer sets listed in Table I. All molecules examined were detected in the cerebrum (lane 1) and sciatic nerve (lane 2). These molecules were not detected in the aorta (lane 3). Only Mdr1a, occludin, and claudin-5 were detected in the kidneys (lane 4).

in TR-BNBs (Fig. 5).

In vivo detection of BBB-forming EC markers in the endoneurium of a rat sciatic nerve

To verify the results of *in vitro* analysis, we examined the expression of tight junction molecules and transporters *in vivo*. RT-PCR experiments indicated that the cerebrum and

Fig. 7. Immunohistochemical staining of tissue sections of the rat cerebrum (A, C, E, G and I) and sciatic nerve (B, D, F, H and J). Endoneurial microvessels (arrows) in the rat sciatic nerve as well as microvessels in the rat cerebrum showed positive staining for p-glycoprotein (A and B), GLUT-1 (C and D), claudin-5 (E and F), occludin (G and H), and claudin-12 (I and J). Scale bars, 100 μ m.

endoneurium of a sciatic nerve express the mRNAs of Mdr1a, GLUT-1, occludin, claudin-5, and claudin-12 (Fig. 6). On the other hand, the aorta expressed none of the molecules and the kidneys expressed only the mRNAs of Mdr1a, occludin, and claudin-5 (Fig. 6). These results indirectly provided the possibility that only ECs in the brain and endoneurium have common fundamental barrier properties and those of other tissues and organs, such as the aorta and kidneys do not. Microvessels in the cerebrum and endoneurium in the sciatic nerve expressed p-glycoprotein, GLUT-1, claudin-5, occludin, and claudin-12 (Fig. 7), corresponding to the detection of the mRNAs of these barrier-related proteins in RT-PCR experiments.

Discussion

Cerebral microvascular endothelial cells forming BBB have TJs that are critical for maintaining low permeability. To date, several proteins have been reported to be localized at TJs including occludin (Furuse *et al.*, 1993), 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 (Bazzoni *et al.*, 2000). Among these proteins, occludin, claudin-5 and ZO-1 have been detected in PnMECs in human sural nerve specimens by immunohistochemistry (Kanda *et al.*, 2004). Hence, it can be expected that these TJ molecules might also play important roles at the cell-cell

boundaries of PnMECs in BNB. Ohtsuki *et al.* reported that the exogenous expression of claudin-5 induces barrier properties in cultured rat brain capillary ECs (Ohtsuki *et al.*, 2007). On the other hand, Nitta *et al.* reported the size-selective opening of BBB in claudin-5-deficient mice (Nitta *et al.*, 2003). In these mice, TJs with a normal appearance are present at the brain capillary endothelial cell-cell contact regions, but leakage of small molecules (<800 Da), although not larger molecules, is observed at BBB. Furthermore, Kanda *et al.* reported that the percentage of claudin-5-positive microvessels in the endoneurium of patients suffering from CIDP was significantly decreased compared with that of patients having noninflammatory neuropathies (Kanda *et al.*, 2004). In the present study, we demonstrated the expressions of TJ components that are common between BMECs and PnMECs. In addition, our results indicated that TR-BNBs, which express claudin-5 at cell-cell boundaries, have significantly higher TEER than HUVECs as well as TR-BBBs, which do not express claudin-5 (Ohtsuki *et al.*, 2007). Interestingly, Honda *et al.* reported that adrenomedullin increased the TEER of rat BMEC monolayer through the expression of claudin-5 (Honda *et al.*, 2006). These data and our results indicate that claudin-5 might be a key molecule in maintaining the integrity of TJs in BNB as well as BBB, and malfunction of this molecule might lead to the breakdown of BNB in many autoimmune peripheral neuropathies.

We would like to determine whether various transporters

functioning at BBB are also present in PnMECs. Although some transporters, for example GLUT1 and p-gp, have been determined to be present on microvessels in the endoneurium by immunohistochemical analyses (Kanda *et al.*, 1997; Saito *et al.*, 1997), no studies have shown the mRNA expression of influx and efflux transporters in PnMECs forming the bulk of BNB. Therefore, we investigated the expression of various influx and efflux transporters in TR-BNBs by RT-PCR analysis. We showed that TR-BNBs expressed GLUT-1, LAT1, and 4Fhc. Rechthand *et al.* (1985) disclosed the facilitated transport of D-glucose from blood into peripheral nerves. Wadhvani *et al.* (1990) demonstrated the facilitated transport of L-phenylalanine across the BNB of rat sciatic nerves. L-phenylalanine is considered to be one of the substrates of system L, which is composed of a heterodimer of the 4F2hc heavy chain and the LAT1 light chain (Kanai *et al.*, 1998). Hence, D-glucose and L-phenylalanine might be preferentially transported from circulating blood into the peripheral nervous parenchyma via GLUT1 and system L in BNB. Recently, creatine has been determined as a major energy-storing source of neuron in the brain and CRT has been reported to play an important role in the uptake of creatine from blood (Ohtsuki *et al.*, 2002). Although there is no direct evidence that peripheral nerves use creatine as an energy-storing source, our results suggest that peripheral nerves also use creatine incorporated via CRT. MCT1 has been reported to be expressed at rat BBB, and supply lactate as an energy source to the brain (Kido *et al.*, 2000). Interestingly, we were not able to detect MCT1 mRNA in TR-BNBs; this indicates that peripheral nerves do not consume lactate as energy even during periods of starvation.

BBB is also involved in the brain-to-blood transport of many drugs and endogenous organic anions via efflux transporters. ABC transporters, such as Mdr1a (p-gp) and Mrp1, mediate brain-to-blood efflux transport coupled with hydrolysis and operate to limit drug permeability to the brain (Terasaki *et al.*, 2003; Pardridge, 2005). It is also well established that ABCG2 functions as a high-capacity efflux transporter of multiple drugs, such as mitoxantrone and doxorubicin, at BBB (Jonker *et al.*, 2005). We demonstrated the mRNA expressions of Mdr1a, Mrp1, and ABCG2 in PnMECs. These results indicate that the nerve fibers of the peripheral nervous system might also be protected against harmful drugs by these efflux transporters. On the other hand, we were not able to detect OAT3 and oatp2 mRNAs in TR-BNBs. Homovanillic acid (HVA) is a major metabolite of dopamine and has been considered to be excreted from the brain to blood by OAT3 (Mori *et al.*, 2003). Dehydroepiandrosterone (DHEAS), a neurosteroid that can interact with GABA type A receptors and sigma receptors, increases memory and learning ability and protects neurons against excitatory amino-acid-induced neurotoxicity (Asaba *et al.*, 2000). Oatp2 has been reported to be involved in the brain-to-blood transport of DHEAS. The absence of OAT3

and oatp2 in TR-BNBs might reflect differences between the environments of CNS and PNS, that is, the former has synapses and the latter does not. Hence, at BNB, as at BBB, many transporters might exist and perform the active influx and efflux transports of required nutrients and potentially harmful substances to peripheral nerves.

We also confirmed the expression of p-glycoprotein and GLUT-1, which are the representative transporters functioning at BBB, and claudin-5, occludin, and claudin-12, which are fundamental TJ proteins at BBB, in endoneurial microvessels *in vivo*. Furthermore, our RT-PCR analysis using rat tissues suggests that only the microvascular endothelial cells of the brain and endoneurium have barrier properties, whereas the endothelium in other organs that do not have barrier systems such as the aorta and kidneys do not.

Altogether, we firstly showed direct evidence that PnMECs in BNB have highly specialized characteristics as barrier-forming cells, sharing TJ molecules and various transporters with BBB-forming endothelial cells. Further analyses to elucidate the common features and different aspects between BNB and BBB are needed because findings of such analyses should lead to the development of novel therapies for many autoimmune peripheral nervous diseases.

Acknowledgments. This work was supported in part by a Neuroimmunological Disease Research Committee grant from the Ministry of Health, Labour and Welfare, Japan and also by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

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(Received for publication, May 9, 2007 and accepted, November 3, 2007)