Conditionally Immortalized Brain Capillary Endothelial Cell Lines Established from a Transgenic Mouse Harboring Temperature-Sensitive Simian Virus 40 Large T-Antigen Gene

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ABSTRACT Five immortalized brain capillary endothelial cell lines (TM-BBB1-5) were established from 3 transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene (Tg mouse). These cell lines expressed active large T-antigen and grew well at 33°C with a doubling time of about 20 to 30 hours. TM-BBBs also grew at 37°C but not at 39°C. However, growth was restored when the temperature of the culture was lowered to 33°C. Although significant amounts of large T-antigen were shown to be present in the cell culture at 33°C, there was less of this complex at 37°C and 39°C. TM-BBBs expressed the typical endothelial marker, von Willebrand factor, and exhibited acetylated low-density lipoprotein uptake activity. The alkaline phosphatase and γ-glutamyltranspeptidase activity in TM-BBBs were -10% and 50% to 80% of brain capillary fraction of normal mice, respectively. D-Mannitol transport in the both apical-to-basal and basal-to-apical directions across the TM-BBB was 2-fold greater than for inulin. TM-BBBs were found to express GLUT-1 but not GLUT-3, and exhibited concentration-dependent 3-O-methyl-D-glucose (3-OMG) uptake activity with a Michaelis-Menten constant of 6.59 ± 1.16 mmol/l. Moreover, P-glycoprotein (P-gp) with a molecular weight of -170 kDa was expressed in all TM-BBBs. Both *mdr* 1a and *mdr* 1b mRNA were detected in TM-BBB4 using reverse transcription-polymerase chain reaction (RT-PCR) analysis. [^{3}H]-Cyclosporin A uptake by TM-BBB was significantly increased in the presence of 100 μmol/l verapamil and vincristine, suggesting that TM-BBB exhibits efflux transport activity via P-gp. In conclusion, conditional brain capillary endothelial cell lines were established from Tg mice. This cell line expresses endothelial markers and transporters at the BBB and is able to regulate cell growth, due to the amount of active large T-antigen in the cell, by changing the culture temperature.

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INTRODUCTION

To date, in vitro cell experiments using freshly isolated cells, primary culture cells, and immortalized

cell lines play a very important role in elucidating the physiological and biological functions of cells. Cultured cells have become increasingly important, not only for studying transport functions for organ-selective drug targeting, but also for toxicological and pharmacological drug screening studies. In order to optimize their usefulness in these fields, it is necessary to develop several types of cell line. The transgenic mouse harboring temperature-sensitive simian virus 40 (ts SV 40) large T-antigen gene (Tg mouse) has several advantages in establishing immortalized cell lines. This is because the Tg mice exhibit stable and identifiable expression of SV 40 large T-antigen gene in all tissues, and cultured cells can be easily immortalized by activation of SV 40 large T-antigen gene at 33°C (1). The cells, which carry ts SV 40 large T-antigen gene product, have very special characteristics. These cells proliferate at 33°C because active large T-antigen binds to tumor suppressor gene product, p53 protein, and/or retinoblastoma (1). However, at temperatures over 37°C large T-antigen becomes unstable and is degraded when cells are cultured (2); thus, the cells stop growing. Several cell lines, such as hepatocyte cells (3), gastric surface mucosal cells (4), and smooth muscle cells (5) have been established from Tg mice and these cell lines exhibit specific differentiated phenotypes. Moreover, this strategy is very useful for establishing cell lines from small organs and tissues, such as brain capillary endothelial cells. It is difficult to make a primary culture of brain capillary endothelial cells from rats or mice due to the fact that these account for only 0.1% to 0.2% of the total cell volume of the brain of these animals (6). Brain capillary endothelial cells from Tg mice have several advantages; these cell lines can be maintained for the expression of active T-anitigen at 33°C and used to study physiological transport functions under conditions involving inactivation of T-antigen at 37°C.

The extracellular matrix plays an important role in the growth of cells in culture. Poly (N-isopropylacrylamide) (PIPAAm), a thermoresponsive polymer, exhibits a lower critical solution temperature of about 32°C in water. PIPAAm is fully hydrated with an extended chain conformation below 32°C, and it is extensively dehydrated and remains compact at temperatures over 32°C (7). Therefore, the cells are cultured on a PIPAAm-coated dish, and detached cells are shifted at 4°C without trypsin. Recently, Kushida et al reported that using PIPAAm for the cell passage of a bovine aortic endothelial cell culture allows the fibronectin matrix to remain adhered to the cell, whereas trypsin treatment destroys the matrix (8). Trypsin-free passage retains in vivo cell functions and avoids damage to the cell membrane when establishing cell lines from Tg mice.

The purpose of the present study is to conditionally establish immortalized brain capillary endothelial cell lines from Tg mice and characterize the endothelial marker, transport activity, and expression of transporter at the blood-brain barrier (BBB).

MATERIALS AND METHODS

Animals

The origin and characteristics of the transgenic mice have been previously described (3). Transgenic mice harboring the ts SV 40 large T-antigen gene (Tg mouse) derived from temperature-sensitive A58 were produced by injection of BamH1 DNA fragments of whole ts SV 40 large T-antigen gene DNA (pSVtsA58) into the pronuclei of fertilized eggs of C57BL/6 mice (Charles River, Yokohama, Japan). The investigations using rats described in this report conformed to the Guidelines of the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan.

Materials

[^{3}H]-3-O-methyl-D-glucose ([^{3}H]-3-OMG, 72.4 Ci/mmol), [^{3}H]-D-mannitol (26.3 Ci/mmol), and [^{14}C]-carboxy inulin ([^{14}C]-inulin, 2.64 mCi/g) were purchased from NEN Life Science Products (Boston, MA); [^{3}H]-cyclosporin A ([^{3}H]-CsA, 8.00 Ci/mmol) was purchased from Amersham Life Science (Buckinghamshire, England); collagenase/dispase, endothelial cell growth factor (ECGF), and fibronectin were purchased from Boehringer Mannheim (GmbH, Mannheim, Germany); dextran (71,000 Da), benzylpenicillin potassium and streptomycin sulfate were purchased from Sigma (St.

Louis, MO); acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL) and acetylated low density lipoprotein (Ac-LDL) were obtained from Biomedical Technologies (Stoughton, MA); and the alkaline phosphatase and γ-glutamyl transpeptidase test kits were obtained from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals were reagent grade and available commercially.

Isolation of brain capillary endothelial cells

The brain capillary-rich fraction was isolated from Tg mice. The cerebrum was excised from Tg mice, dissected into 2-mm pieces, and homogenized in a Potter-Elvehjem homogenizer (Wheaton, Millville, NJ). Homogenate was added to the same volume of 32% dextran, resulting in a 16% dextran solution, and then centrifuged (4,500*g*, 15 minutes, 4°C). The resulting capillary-rich pellet was incubated in 0.1% collagenase/dispase for 3 hours at 37°C. Brain capillary endothelial cell culture was performed using a modification of the procedure described by Ichikawa et al (9). The brain capillary endothelial cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 15 ng/ml ECGF, 100 U/ml benzyl penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 10 U/ml heparin, and 20% fetal bovine serum (FBS) (Moregate, Bulimba, Australia). Cells were seeded onto 35-mm fibronectin-treated dishes (Falcon 3001; Becton Dickinson, Lincoln Park, NJ) which were coated with poly(N-isopropylacrylamide) (PIPAAm) (8) and cultured at 37°C for the first 48 to 72 hours to allow the cells to attach to the dish. Cells were subsequently cultured at 33°C in a humidified atmosphere of 5% CO_2 and 95% air. After appropriate intervals, cells were detached at 4°C for 2 hours and treated with 0.1% collagenase/dispase at 37°C for 5 minutes to obtain separate single cells. Following two or three passages, cells were cloned from a single cell by colony formation and isolated twice from other cells using a penicillin cup.

Measurement of growth kinetics

Cells (1.8 X 10^4 cells/well) were cultured on collagen-coated 12 well plates (Becton Dickinson) at 33, 37, or 39°C. After a predetermined time period, cells were trypsinized, and counted in a hemocytometer. The doubling time during the logarithmic growth phase was estimated by fitting the cell number versus the culture time data to a model using a nonlinear least-squares regression analysis program (MULTI) (10).

Western blot analysis

The membrane protein fraction of immortalized brain capillary endothelial cell lines (TM-BBBs) and brain capillary for GLUT-1, GLUT-3, and P-glycoprotein (P-gp) was prepared using a modification of the procedure of Kitagawa et al (11) and Ichikawa et al (12). The mouse brain capillary fraction, used as a positive control, was isolated from normal mice as described above. Deglycosylation was carried out by incubating the membrane protein fraction with *N*-glycosidase F (Boehringer Mannheim) for 30 minutes at 37°C. The TM-BBB cells for large T-antigen were lysed in ice-cold phospate-buffered saline (PBS) containing 3% sodium dodecyl sulfate (SDS) and protease inhibitors (83 μmol/l antipain, 73 μmol/l pepstatin A, and 0.1 mmol/l leupeptin). The protein (10 to 100 μg) was electrophoresed on SDS-polyacrylamide gel and subsequently electrotransferred to a nitrocellulose membrane (Toyo Roshi, Tokyo, Japan). The membranes were incubated with mouse anti-SV 40 large T-antigen monoclonal antibody (1:100) (Calibiochem, Cambridge, MA), rabbit anti-GLUT-1 antibody (1:2000) (Chemicon, Temecula, CA), rabbit anti-GLUT-3 antibody (1:5000) (Charles River, Southbridge, MA), and mouse anti-P-gp monoclonal antibody (1:100) (C219; Signet, Dedham, MA) as the primary antibody, and with HRP conjugated anti-mouse or rabbit IgG (Chemicon) as the secondary antibody, before detection of luminescence (ECL; Amersham, Downer Grove, IL).

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was prepared from PBS-washed cells using Trizol reagent (Gibco BRL, Rockville, MD). Reverse transcription (RT) and polymerase chain reaction (PCR) amplification were carried out with GeneAmp equipment (PCR system 9700; Perkin-Elmer, Norwalk, CT). Single-strand cDNA was synthesized from 1 μg RNA by reverse transcription (ReverTra Ace; Toyobo, Osaka, Japan) using an oligo dT primer. The sequences of the specific primers were as follows: the sense primer 5'-CCCTGCCTCATCAATGAGTGTGT-3' and the antisense primer 5'-CGCTTCAGTGTCATGATCTGTCC-3' for von Willebrand factor; the sense primer 5'-CCAGCAGTCAGTGTGCTTACA-3' and the antisense primer 5'-CATAAGTGGGAGCGCCAC-3', for mdr 1a; the sense primer 5'-GCTGTTGGCG TATTTGGG-3' and the antisense primer 5'-AGCATCAAGAGGGGAAGTAATG-3' for mdr 1b; and the sense primer 5'-TGATGACATCAAGAAGGTGGTGAAG-3' and the antisense primer 5'-TCCTTGGAGGCCATGTAGGCCAT-3' for the GAPDH. PCR was performed with von Willebrand factor-specific primer through 40 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute and mouse mdr 1a, mdr 1b, and GAPDH-specific primer through 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 1 minute. The PCR products were separated by electrophoresis in agarose gel and visualized with imaging equipment (EPIPRO 7000; Aisin, Aichi, Japan) in the presence of ethidium bromide. These products were then cloned into a plasmid vector using the p-GEM-T Easy Vector System I (Promega, Madison, WI) and sequenced by DNA sequencer (Model 4200; Li-COR, Lincoln, NE).

Uptake of Dil-Ac-LDL

TM-BBBs were cultured on a collagen-coated cover-glass (Becton Dickinson) at 33°C for 48 hours. The cells were then incubated with DMEM containing 2% bovine serum albumin (BSA) and 10 μg/ml Dil-Ac-LDL in the presence and absence of 200 μg/ml unlabeled Ac-LDL at 37°C or 4°C for 4 hours. Cells were washed five times with PBS and fixed in 3% formaldehyde-PBS at room temperature for 20 minutes. The cover-glass was mounted in glycerol and viewed at the rhodamine excitation/emission wavelength using Leica confocal laser scanning microscope (TCS SP, Heidelberg, Germany).

Uptake study for 3-OMG and CsA in TM-BBBs

TM-BBB cells (3 X 10^5 cells/well) were cultured on 24 well plates (Iwaki, Tokyo, Japan) for 24 hours at 33°C. After removal of medium, cells were washed with ECF buffer per liter (122 mmol NaCl, 3 mmol KCl, 0.4 mmol K_2HPO_4, 25 mmol $NaHCO_3$, 1.4 mmol $CaCl_2$, 1.2 mmol $MgSO_47H_2O$, 10 mmol HEPES, 10 mmol glucose, pH 7.4, 290 mOs/kg) for CsA uptake and D-glucose-free ECF buffer for 3-OMG uptake. Uptake was initiated by applying [^{3}H]-3-OMG (1.25 μCi/well) and [^{14}C]-inulin (0.25 μCi/well) or [^{3}H]-CsA (0.2 μCi/well) and [^{14}C]-inulin (0.04 μCi/well) at 37°C. [^{14}C]-Inulin was used to estimate the volume of water adhering. After a predetermined time period, uptake was terminated by removing the applied solution and immersing the cells in ice-cold ECF buffer. The cells were then solubilized in 1 ml 1% Triton X-100 solution. Fifteen microliters of this solution was taken for protein assay using the DC protein assay kit (Bio-Rad, Hercules, CA) with BSA as a standard. The remainder of the sample was mixed with 5 ml scintillation cocktail (Hionic-fluor; Packard, Meriden, CT) and radioactivity was measured using a liquid scintillation counter equipped with an appropriate crossover correction for [^{3}H] and [^{14}C] (LS 6500; Beckman, Fullerton, CA).

Transport study for D-mannitol and inulin in TM-BBB

TM-BBB4 cells (5 X 10^4 cells/cm^2) were cultured on a collagen-coated Transwell insert (pore size 0.4 μm; Corning Coster, Cambridge, MA) for 48 hours at 33°C. After removal of the medium, the cells were washed with ECF buffer, and 0.5 ml and 1.5 ml ECF buffer were added to the apical and basal side, respectively. Following incubation at 37°C, transport was initiated by applying [^{3}H]-D-mannitol (3 μCi/ml)

and [^{14}C]-inulin (0.6 μCi/ml). The ECF buffer (100 μl) from the receiver side chamber was collected and replaced with fresh ECF buffer at 2, 5, 10, 30, and 60 minutes. The sample was then mixed with 5 ml scintillation cocktail (Hionic-fluor) to measure the radioactivity in a liquid scintillation counter (LS 6500; Beckman).

Data analysis

For kinetic studies, the Michaelis-Menten constant (K_m) and the maximal rate (V_{max}) of 3-OMG uptake were calculated from the following equation using MULTI (10):

$$V = V_{max} X C / (K_m + C) \quad (Eq. 1)$$

where V and C are the uptake rate of 3-OMG at 20 seconds and the concentration of 3-OMG, respectively.

Unless otherwise indicated, all data represent means ± SEM. An unpaired two-tailed Student's t test was used to determine the significance of any differences between two group means. Statistical significance among means of more than two groups was determined by a one-way analysis of variance (ANOVA) followed by the modified Fisher's least squares difference method.

RESULTS

Endothelial characterization of TM-BBBs

Five immortalized mouse brain capillary endothelial cell lines (TM-BBBs) were obtained from three Tg mice. Morphologically, TM-BBB4 appeared to be spindle-fiber shaped (Figure 1A). TM-BBBs were brightly stained after incubating with Dil-Ac-LDL for 4 hours at 37°C, and a typical result for TM-BBB4 is shown in Figure 1B. An X-Z section showed that fluorescence was located in the cytoplasm and cell wall, but not in the cell nucleus (Figure 1C). The intensity of fluorescence was reduced in the presence excess (200 μg/ml) unlabeled Ac-LDL (Figure 1D and E) or at 4°C (data not shown), indicating specific uptake. Expression of the von Willebrand factor gene in TM-BBBs was investigated by RT-PCR.

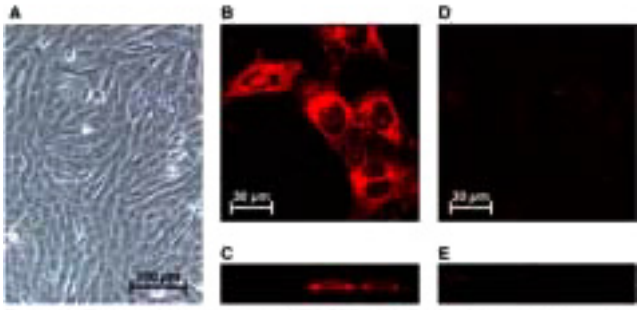

Figure 1. Phase microscopic image of TM-BBB4 (A). Confocal laser scanning microscopic image of DiI-Ac-LDL uptake by TM-BBB4 in the X-Y section (B) and X-Z section (C), and in the presence of unlabeled Ac-LDL in the X-Y section (D) and X-Z section (E). A: TM-BBB4 appears to have a spindle-fiber shape morphology. B, C: DiI-Ac-LDL (10 μg/ml) uptake by TM-BBBs was performed at 37°C for 4 hr. D, E: DiI-Ac-LDL (10 μg/ml) uptake by TM-BBBs was performed at 37°C for 4 hr in the presence of unlabeled Ac-LDL (200 μg/ml).

Using primers specific for the von Willebrand factor gene, a band of product of the expected size (410 bp) was amplified from the lung (lane 1) and cultured TM-BBB RNA (lane 2-6) (Figure 2).

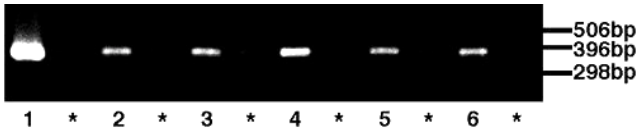

Figure 2. RT-PCR analysis of von Willebrand factor in TM-BBBs. Lane 1; lung as a positive control, lane 2; TM-BBB1, lane 3; TM-BBB2, lane 4; TM-BBB3, lane 5; TM-BBB4, lane 6; TM-BBB5. * ; Respective RT(-) for left side lane.

DNA sequencing of the bands demonstrated greater than 80% homology with von Willebrand factor gene of canine (Genbank accession number L76227), human (13), and porcine (14) origin; mouse von Willebrand factor mRNA has not been cloned yet.

The alkaline phosphatase activity in TM-BBBs ranged from 4.62 to 17.6 mU/mg protein and this activity accounted for 2.43% to 9.26% of the mouse brain capillary-rich fraction (Table 1). On the other hand, the γ-glutamyl transpeptidase activity ranged from 3.00 to 4.53 mU/mg protein and accounted for 52.1% to 77.2% of the mouse brain capillary-rich fraction (Table 1).

Temperature-dependence of cell growth and expression of T-antigen

TM-BBBs expressed a large T-antigen with a molecular weight of 94 kDa, which has the same molecular weight as the COS-1 cells used as a positive control (15) (lane 8, Figure 3A).

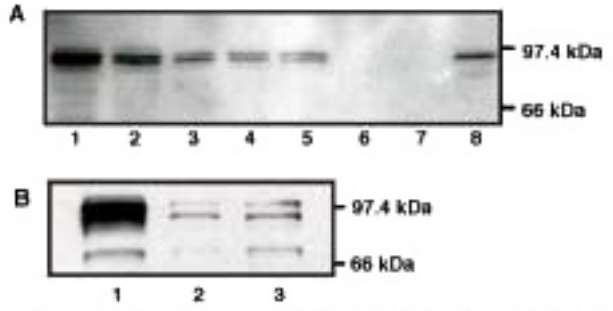

Figure 3. Western blot analysis of large T-antigen in TM-BBBs at 33°C (A) and effect of temperature on expression of large T-antigen in TM-BBB4 (B). A: lane 1; TM-BBB1, lane 2; TM-BBB2, lane 3; TM-BBB3, lane 4; TM-BBB4, lane 5; TM-BBB5, lane 6; mouse brain capillary fraction, lane 7; mouse parenchymal fraction, lane 8; COS-1 cell as a positive control. B: lane1; 33°C culture for 4 days, lane 2; 33°C culture for 2 days and followed by 37°C culture for 2 days, lane 3; 33°C culture for 2 days and followed by 39°C culture for 2 days.

Growth of TM-BBBs was measured at 33°C (Figure 4A), 37°C, and 39°C (Figure 4B).

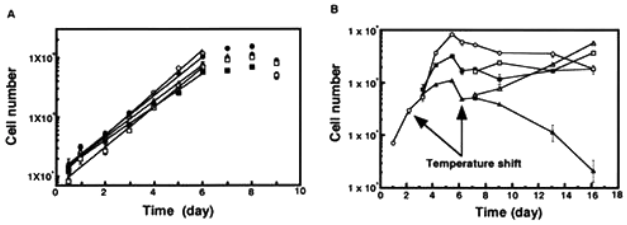

Figure 4. Cell growth of TM-BBBs at 33°C (A) and effect of temperature on cell growth (B) in TM-BBB4. Each point represents the mean ± S.E.M.(n=3). A: □; TM-BBB1, ●; TM-BBB2, △; TM-BBB3, ○; TM-BBB4, ■; TM-BBB5. B: ○; □; △ at 33°C, ■; 37°C, ▲; 39°C.

TM-BBBs proliferated at 33°C (permissive temperature) (Figure 3A) with a doubling-time of 20 to 30 hours TM-BBB4 exhibited the fastest growth with a doubling-time of 19.5 ± 1.0 hours. TM-BBB4 also proliferated at 37°C (intermediate temperature) (Figure 4B). At the nonpermissive temperature (39°C), TM-BBB4 did not grow, but growth resumed when the culture temperature was lowered to 33°C (Figure 4B). When TM-BBB4 was cultured at 37°C or 39°C for 2 days, the amount of large T-antigen was lower than that at 33°C (Figure 3B). These results indicate that T-antigen is inactivated at non-permissive temperatures.

Analysis of hexose transport in TM-BBBs

The expression of GLUT-1 in TM-BBBs was analyzed using anti-GLUT-1 antibody. A band at -55 kDa was found in TM-BBB4 (lane 3), and this had the same molecular weight as the mouse brain capillary-rich fraction used as a positive control (lane 1) (Figure 5A). After deglycosylation, the bands shifted to -40 kDa in the brain capillary-rich fraction and TM-BBB4. However, deglycosylation did not alter the bands at more than 66 kDa in TM-BBB4, indicating nonspecific binding.

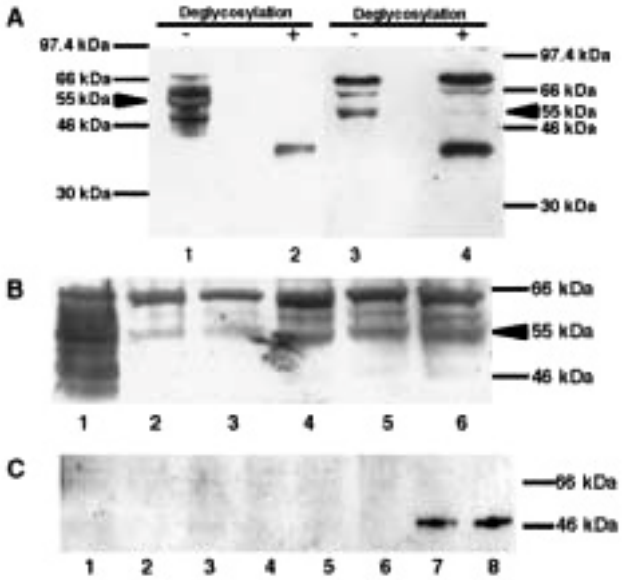

Figure 5. Western blot analysis of GLUT-1 (A and B) and GLUT-3 (C). A: lane 1; mouse brain capillary-rich fraction as a positive control without *N*-glycosidase F, lane 2; mouse brain capillary-rich fraction as a positive control with *N*-glycosidase F, lane 3; TM-BBB4 without *N*-glycosidase F, lane 4; TM-BBB4 with *N*-glycosidase F. B: lane 1; mouse brain capillary-rich fraction as a positive control, lane 2; TM-BBB1, lane 3; TM-BBB2, lane 4; TM-BBB3, lane 5; TM-BBB4, lane 6; TM-BBB5. C: lane 1; mouse brain capillary-rich fraction, lane 2; TM-BBB1, lane 3; TM-BBB2, lane 4; TM-BBB3, lane 5; TM-BBB4, lane 6; TM-BBB5, lane 7; mouse brain parenchymal fraction as a positive control, lane 8; mouse brain homogenate as a positive control.

TM-BBBs exhibited a band at -55 kDa, which is the same molecular weight as the brain capillary-rich fraction) (Figure 5B). No GLUT-3 was detected in TM-BBBs (lane 2-6) or the brain capillary-rich fraction (lane 1), while the mouse brain parenchymal fraction and brain homogenate used as positive controls (lanes 7 and 8) (16), expressed GLUT-3 (Figure 5C). Figure 6A shows the time-courses of [^{3}H]-3-OMG uptake in the TM-BBBs.

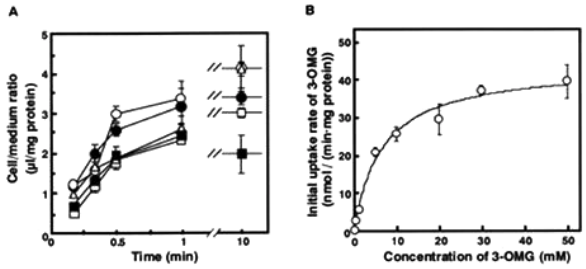

Figure 6. Time-courses of [^{3}H]-3-OMG uptake by TM-BBBs (A) and concentration-dependence of 3-OMG uptake by TM-BBB4 (B). Each point represents the mean ± S.E.M.(n=4-8).
A: □; TM-BBB1, ●; TM-BBB2, △; TM-BBB3, ○; TM-BBB4, ■; TM-BBB5. B: The K_m is 6.59 ± 1.16 mM and V_{max} is 43.6 ± 2.2 nmol/(min·mg protein).

The initial uptake rate determined from the slope of the 3-OMG uptake from 10 to 30 seconds was 2.63 to 5.30 µl/(min · mg protein) in TM-BBBs. Of the TM-BBBs, TM-BBB4 was the most abundant and its initial uptake rate was 5.30 ± 1.98 µl/(min · mg protein). [^{3}H]-3-OMG uptake by TM-BBB4 took place in a concentration-dependent manner with a K_m of 6.59 ± 1.16 mmol/l and a V_{max} of 43.6 ± 2.2 nmol/(min · mg protein) (mean ± S.D.) Figure 6B.

Analysis of transcellular transport in TM-BBB

Transendothelial electrical resistance (TEER) was measured using Millicell-ERS equipment (Milipore, Bedford, MA) at 33°C. The TEER in TM-BBB1-5 was 105 to 118 ohm · cm^2 48 hours after seeding (1 X 10^3 cells/cm^2) on a collagen-coated Transwell (Corning Coster). The time-courses of D-mannitol (182 Da) and inulin (5,000 Da) transport across the TM-BBB4 monolayers in both the apical-to-basal (a→b) and basal-to-apical (b→a) directions are shown in Figure 7.

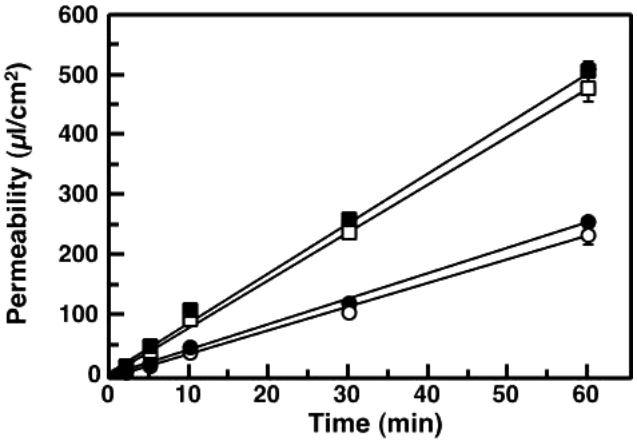

Figure 7. Time-courses of [^{3}H]-D-mannitol and [^{14}C]-inulin transport across TM-BBB4. Each point represents the mean ± S.E.M. (n=3). TM-BBB4 cells were cultured on collagen-coated Transwell. [^{3}H]-D-mannitol transport in the apical-to-basal (■) and basal-to-apical (□) directions and [^{14}C]-inulin transport in the apical-to-basal (●) and basal-to-apical (○) directions were performed at 37°C.

The permeability coefficient (Papp) of D-mannitol in the a→b and b→a directions was 13.9 X 10^{-5} ± 0.4 X 10^{-5} cm/sec and 13.2 X 10^{-5} ± 0.7 X 10^{-5} cm/sec, respectively. The Papp of inulin in the a→b and b→a directions was 7.06 X 10^{-5} ± 0.26 X 10^{-5} cm/sec and 6.50 X 10^{-5} ± 0.50 X 10^{-5} cm/sec, respectively. There was no significant difference between Papp in the a→b and b→a directions for both solutes. D-Mannitol transport in both directions was about 2-fold greater than that of inulin.

Analysis of efflux transport in TM-BBBs

The expression of P-gp in TM-BBBs was detected with C219 as shown in Figure 8A.

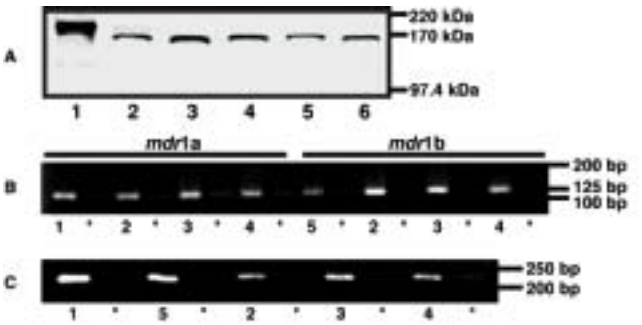

Figure 8. Western blot analysis of P-glycoprotein in TM-BBBs (A) and RT-PCR analysis of TM-BBB4 for *mdr* 1a, *mdr* 1b (B), and GAPDH (C).
A: lane 1; mouse brain capillary-rich fraction as a positive control, lane 2; TM-BBB1, lane 3; TM-BBB2, lane 4; TM-BBB3, lane 5; TM-BBB4, lane 6; TM-BBB5. B, C: lane 1; small intestine as a positive control for *mdr* 1a, lane 2; brain capillary-rich fraction as a positive control for *mdr* 1a, lane 3; 33°C culture, lane 4; 33°C culture for 2 days and followed by 37°C culture for 2 days, lane 5; kidney as a positive control for *mdr* 1b. * ; respective RT(-) for left side lane

The bands in TM-BBBs were detected at -170 kDa. The band in the brain capillary-rich fraction used as a positive control was found at -180 kDa (lane 1). RT-PCR experiments were performed to examine whether mdr 1a, mdr 1b, and GAPDH genes (used as a control) were expressed in TM-BBB4. Using primers specific for mdr 1a resulted in the amplification of an expected 110 bp fragment with total RNA prepared from mouse small intestine (lane 1), brain capillary-rich fraction (lane 2) as a positive control (17,18) and TM-BBB4 cultured at 33°C and 37°C (lane 3 for culture at 33°C and lane 4 for culture at 37°C) (Figure 8B). In the case of culture at 37°C, TM-BBB4 was cultured at 33°C for 2 days and then cultured at 37°C for 2 days. With primers specific for mdr 1b, a fragment corresponding to the expected size (113 bp) was amplified from RNA prepared from kidney as a positive control (17) (lane 5), brain capillary-rich fraction (lane 2) and TM-BBB4 at 33°C and 37°C (lane 3 for culture at 33°C and lane 4 for culture at 37°C) (Figure 8B). GAPDH gene was determined as a house-keeping gene for the mouse brain capillary-rich fraction, small intestine, kidney, and TM-BBB4 cultured at 33°C and 37°C (Figure 8C). The DNA sequence of the bands was identical to

mdr 1a and 1b, with homologies of 99% (19) and 100% (20), respectively. Figure 9A shows the time-course of [^{3}H]-CsA uptake by TM-BBB4 at 37°C.

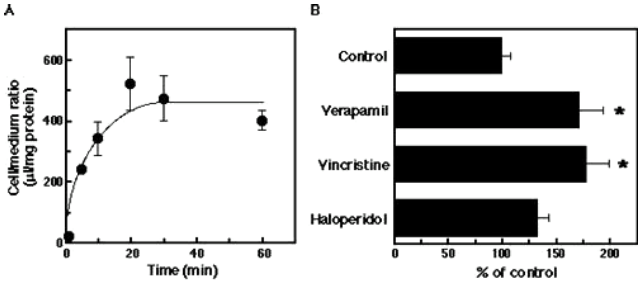

Figure 9. Time-course of [^{3}H]-cyclosporin A (CsA) uptake (A) and effects of several compounds on the [^{3}H]-CsA uptake by TM-BBB4 (B).
A: Each point represents the mean ± S.E.M. (n=4).
B: Each column represents the mean ± S.E.M. (n=8-10). After cells were pretreated at 37°C for 30 min in the presence of each compound (100 μM), [^{3}H]-CsA uptake was measured in the presence of each compound at 37°C for 30 min. *p<0.05, significantly different from control.

The accumulation of [^{3}H]-CsA was time-dependent and reached a steady state after 20 minutes. The steady-state uptake of [^{3}H]-CsA increased significantly, by 172% and 178%, in the presence of 100 μmol verapamil and vincristin, respectively. These agents are P-gp inhibitors (21), whereas 100 μmol haloperidol, used as a negative control, had no effect on [^{3}H]-CsA uptake.

DISCUSSION

In the present study, conditionally immortalized brain capillary endothelial cell lines (TM-BBBs) were established from transgenic mice harboring ts SV 40 large T-antigen gene, and these grew well at 33°C. TM-BBBs have a spindle-fiber shaped morphology (Figure 1) and possess von Willebrand factor mRNA (Figure 2) for an endothelial-specific marker (22), a receptor for Ac-LDL uptake as an endothelial and macrophage marker (23) (Figure 1), and 52 to 77% of the γ-glutamyl transpeptidase activity of the brain capillary-rich fraction morphology (Table 1). TM-BBBs were of cerebral origin and cloned from a single cell by means of colony formation and penicillin cup isolation. Therefore, these findings suggest that TM-BBBs are brain capillary endothelial cell lines free from contamination by other cells.

The Tg mouse is very useful for establishing an immortalized cell line for small organs and tissues, such as brain capillary endothelial cells, because ts large T-antigen gene is ubiquitously expressed in the Tg mouse (3). Moreover, it is not necessary to purify cells at the first cell isolation step. Cells can be cloned from different types of cells, such as fibroblasts and pericytes, during colony formation and penicillin cup isolation. The Tg mouse develops as a wild type until it is 5 months old (1). Thus, the large T-antigen gene is unlikely to disrupt any other critical gene in the cell. TM-BBBs, hepatocyte cell lines (3), and gastric surface mucous cell lines (4) from the Tg mouse exhibit stable growth and differentiated characteristics more than 6 months after cloning. Compared with the primary culture of bovine brain capillary endothelial cells (BCEC) (21,24), TM-BBB4 cells grew rapidly with a doubling-time of about 20 hours at 33°C, which is a permissive temperature for activation of ts large T-antigen (Figure 3A and 4A). At a nonpermissive temperature (39°C), there was a reduction in the number of TM-BBB4 cells (Figure 4B) because apoptosis can be induced by an increase in free p53 proteins (2) liberated from the complex with large T-antigen due to degradation (Figure 3B). At an intermediate temperature (37°C), the cell number remained unchanged after a temperature shift from 33°C (Figure 4B) because although the amount of the active form of large T-antigen at 37°C was reduced, there may have been enough to maintain cell growth without inducing apoptosis. Temperature-conditionally immortalized brain capillary endothelial cells, TM-BBBs are not only very easy to handle but are also useful for investigating transport functions under normal conditions at the BBB based on cellular and molecular aspects. Our preliminary experiments suggest that the mdr 1a expression in TM-BBB4 at 37°C for 4 days is increased 2-fold, but mdr 1b expression falls by 50% compared with that following culture at 33°C using quantitative RT-PCR (ABI 7700 Sequence Detector; Perkin-Elmer). Indeed, the smooth muscle cell line established from the Tg mouse expressed more protein and higher mRNA levels of smooth muscle myosin heavy chain-1 and calponin as specific smooth muscle markers at 39°C than was the case at 33°C. These results suggest that SV 40 large T-antigen and/or p53 affect the expression of smooth muscle-specific markers (5).

The TEER values for TM-BBB1-5 were 105 to 118 ohm · cm^2 , which is comparable with the value for

primary culture of rat brain capillary endothelial cells (25). The Papp for D-mannitol and inulin was 13.9 X 10^{-5} cm/sec and 7.06 X 10^{-5} cm/sec, respectively, and these figures are relatively greater than that of sucrose (362 Da, 5.1 X 10^{-3} cm/min = 8.5 X 10^{-5} cm/sec) in BCEC (6). It is not tight enough for a paracellular transport study compared with the TEER value in vivo (1,500 to 8,000 ohm · cm^2) (26,27). However, the expression of tight-junction strand protein mRNAs, such as occludin, junctional adhesion molecule, and claudin-5 (28), was found by RT-PCR (T. Takashima, K. Hosoya, and T. Terasaki, unpublished observations). It is possible to study the regulation of tight-junction formation since these tight-junction strand proteins, especially occludin, are associated with the tight-junction in brain endothelial cells (29).

GLUT-1 was expressed at -55 kDa in TM-BBBs as well as the mouse brain capillary-rich fraction (Figure 5B). N-Glycosidase F treatment showed that the size shifted both in TM-BBB4 and the mouse brain capillary-rich fraction was almost the same at -40 kDa, supporting GLUT-1 expression (30). TM-BBB4 exhibits 3-OMG uptake activity with a K_m of 6.59 mmol/l and a V_{max} of 43.6 nmol/(min · mg protein), suggesting that GLUT-1 plays a role in D-glucose uptake. Compared with in vivo functions involving D-glucose uptake clearance (CL), the conditions required are: 1) the amount of protein for TM-BBB is 22 cm^2/mg protein (data not shown), 2) the surface area of the mouse cerebrum capillary is similar to that in rat, ie 100 cm^2/g brain (6). From this, the BBB uptake CL of 3-OMG is estimated to be 30.0 µl/(min · g brain) according to CL= V_{max}/K_m [=43.6 nmol/(min · mg protein)÷22 cm^2/mg protein X 100 cm^2/ g brain÷6.59 mmol/l]. Cornford et al used the brain uptake index method and reported that D-glucose uptake by mouse brain across the BBB had a K_m of 6.67 mmol/l, a V_{max} of 1,562 nmol/(min · g brain), and a resulting CL of 234 µl/(min · g brain) (31). TM-BBB, therefore, exhibits at least 1/8 the D-glucose transport activity of GLUT-1 in vivo.

Both Western blot and RT-PCR analyses suggest that P-gp encoded mdr 1a and mdr 1b are expressed in TM-BBB4 (Figure 8). Although TM-BBBs expressed P-gp at -170 kDa (32), the brain capillary-rich fraction expressed P-gp at -180 kDa, as reported by Lechardeur et al. (33). A different form of glycosylation may take place in TM-BBBs and mouse brain capillary endothelial cells. The mouse BBB in vivo expresses mdr 1a, but may not express mdr 1b (18). It is possible that mdr 1b is up-regulated in the culture cells. The mouse brain capillary endothelial cell line (MBEC) has only mdr 1b (32) while the rat brain endothelial cell line, RBE4, which is a transfected cell line from SV 40 T-antigen gene, expresses both mdr 1a and 1b although isolated rat brain capillary endothelial cells have been shown to express mdr 1a by means of RT-PCR (34). This does not agree with our results. In addition, mdr 1b was also detected in the mouse brain capillary-rich fraction (Figure 8B), and it is possible to detect mdr 1b from brain parenchymal cells due to the identification of mdr 1b in mouse whole brain by RNase protection analysis (17).Further studies are necessary to investigate whether mdr 1b is expressed in mouse brain capillary and regulates mdr 1b expression during the culture period. TM-BBB4 exhibits efflux transport activity for CsA since CsA cell accumulation in TM-BBB4 was enhanced in the presence of verapamil and vincristin, typical P-gp inhibitors (21) (Figure 9B). TM-BBB4 provides P-gp which is capable of efflux transport activity and at least part of the P-gp in TM-BBB4 may be derived from the P-gp gene in vivo.

In conclusion, conditionally immortalized brain capillary endothelial cell lines, TM-BBBs, were established using the Tg mouse and trypsin-free passage. This cell line can be used to regulate cell growth by changing the culture temperature. TM-BBB4 provides GLUT-1 and P-gp which are capable of 3-OMG transport and CsA efflux transport, respectively. This strategy will be useful for establishing cell lines for small organs and tissues and could be applied to transport studies of drugs, neurotransmitters, and their metabolites at the BBB.

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