Pharmaceutical Research, Vol. 24, No. 4, April 2007 (© 2007)
DOI: 10.1007/s11095-006-9188-x

Research Paper

Establishing a Method to Isolate Rat Brain Capillary Endothelial Cells by Magnetic Cell Sorting and Dominant mRNA Expression of Multidrug Resistance-associated Protein 1 and 4 in Highly Purified Rat Brain Capillary Endothelial Cells

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Received September 5, 2006; accepted November 6, 2006; published online February 22, 2007

Purpose. To establish a method for isolating highly purified brain capillary endothelial cells (BCECs) from rat brain by using magnetic cell sorting, and clarify the expression levels of multidrug resistance-associated protein (Mrp) subtypes in these highly purified BCECs.
Methods. The cells were prepared from the capillary enriched-fraction by enzyme digestion, and reacted with anti-PECAM-1 antibody. The cell sorting was performed by autoMACS. The mRNA levels were measured by quantitative real-time PCR analysis.
Results. From five rats, 2.3×10^6 cells were isolated in the PECAM-1(+) fraction and the percentage of labeled cells in this was 85.9%. PECAM-1, claudin-5 and Tie-2 mRNA were concentrated in the PECAM-1(+) fraction compared with rat brain. The contamination by neurons and astrocytes was markedly less than in the brain capillary fraction prepared by the glass bead column method. Mrp1 and 4 were predominantly expressed in the PECAM-1(+) fraction at similar levels to Mdr1a. The mRNA levels of Mrp5 and 3 were 10.6 and 7.60% of that of Mrp1, respectively.
Conclusions. This new purification method provides BCECs with less contamination by neural cells. In the isolated BCECs, Mrp1 and 4 are predominantly expressed, suggesting that they play an important role at the rat blood-brain barrier.

KEY WORDS: brain capillary endothelial cells; magnetic cell sorting; multidrug resistance-associated protein; PECAM-1; purification.

INTRODUCTION

The multidrug resistance-associated protein (Mrp/ABCC) subfamily is a member of the ATP binding cassette (ABC) transporter superfamily and actively transports substrates out of the cell using the energy from ATP hydrolysis (1). Since subtypes of the Mrps, such as Mrp1, 4, 5 and 6, have been reported to be expressed in brain capillary endothelial cells (BCECs) (2), Mrps are believed to be able to limit drug distribution into the brain as P-glycoprotein (Mdr1/ABCB1) does. Indeed, in previous studies using deficient animals, Mrp1, 2 and 4 were suggested to limit the brain distribution of 17β-estradiol-D-17β-glucuronide ($E_2$17βG), phenytoin and topotecan, respectively (3–5).

Mrps mediate the transport of organic anions, glucuronide-conjugated compounds and nucleoside analogs, while Mdr1 transports lipophilic and cationic compounds, suggesting that MRPs are involved in a type of blood-brain barrier (BBB) efflux transport different from that by Mdr1 (1, 6). Therefore, a knowledge of the contribution of each Mrp subtype to the BBB transport system is important to properly understand drug distribution across the BBB. To address this issue, a comparison of drug distribution into the brain between deficient animals and wild-type animals is a reasonable approach. However, in Mrp2-deficient rats, induction and suppression of other Mrp subtypes have been reported (7, 8), and such compensatory effects cannot be ruled out in a study using deficient animals. For this reason, the involvement of subtypes in the BBB efflux transport should be, initially, assessed by a comparison of the expression levels of each Mrp subtype in BCECs.

The expression levels of Mrp subtypes in BCECs have been reported using isolated brain capillaries and/or cultured BCECs (2, 9–11). Zhang *et al.* (2) have found Mrp1, 4 and 5

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ABBREVIATIONS: 6-mp, 6-mercaptopurine; ABC, ATP binding cassette; AZT, azidothymidine; BBB, blood-brain barrier; BCECs, brain capillary endothelial cells; DHEAS, dehydroepiandrosterone sulfate; $E_2$17βG, 17β-estradiol-D-17β-glucuronide; FL2, fluorescence channel 2; Mrp, multidrug resistance-associated protein; PBS, phosphate buffered saline; PE, Phycoerythrin; PECAM-1, platelet endothelial cellular adhesion molecule-1; SSC, side scattered light.

mRNAs in primary cultured bovine BCECs and isolated bovine capillaries, while Mrp6 was detected only in primary cultured BCECs. This difference illustrates the difficulties in comparing the expression levels of Mrp subtypes at the BBB. The isolated capillaries are hard to purify from neural cells, and mRNA expression of Mrps has been detected in primary cultured neurons and astrocytes (12). Although primary cultured cells are less contaminated, the expression of transporters is changed during *in vitro* culture. Induction of Mrp1 mRNA expression during culture has been reported in human and porcine BCECs (9, 10). Therefore, the expression of Mrp subtypes at the BBB should be determined in highly purified freshly isolated BCECs.

The purpose of the present study was, therefore, initially to isolate highly purified rat BCEC by magnetic cell sorting using vascular endothelial cell selective surface antigen, platelet endothelial cellular adhesion molecule-1 (PECAM-1/CD31) (13), and then to clarify the mRNA expression level of each subtype of Mrps in the purified BCECs.

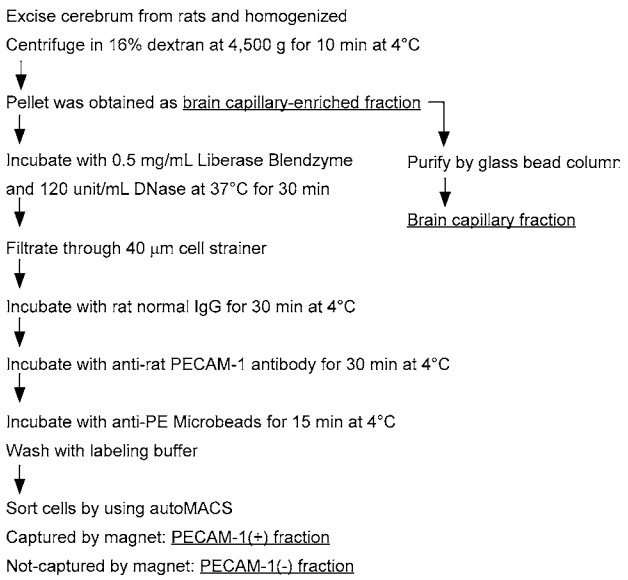

Fig. 1. Isolation procedure for brain capillary endothelial cells using magnetic cell sorting.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 180–200 g, were purchased from Charles River (Yokohama, Japan). All experiments were approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Isolation of the Brain Capillary Fraction by the Glass Bead Column Method

Rat brain capillaries were isolated using a modification of the procedure of Boado and Pardridge (14). Briefly, the cerebrum excised from five rats was dissected into pieces, and homogenized in four volumes (v/w) of phosphate buffered saline (PBS) using 11 strokes of a glass–teflon homogenizer. The homogenate was added to the same volume of 32% dextran solution, and homogenized with three strokes. This homogenate was centrifuged (4,500 g, 10 min, 4°C) and the resulting pellets and supernatant were collected. The supernatant were added to the same volume of 32% dextran solution, and centrifuged again. The resulting pellets were mixed and washed in PBS to obtain the brain capillary-enriched fraction (Fig. 1). This fraction was resuspended in buffer A (100 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 0.91 mM KH_2PO_4, 1.2 mM $MgSO_4$ $7H_2O$, 25 mM $NaHCO_3$, 10 mM glucose, 1.0 mM Pyruvate, 15 mM Hepes, 0.5% (w/v) bovine serum albumin, pH7.4). The suspension was filtered through an 85 μm-nylon mesh. The filtrate was passed over a column containing 350–500 μm glass beads, and, after washing with 40 ml buffer A, the capillaries adhering to the beads were collected by gentle agitation. The capillaries were centrifuged, and the pellets were washed in PBS to obtain the brain capillary fraction (Fig. 1).

Isolation of BCEC by Magnetic Cell Sorting

The brief procedure is shown in Fig. 1. To prepare a single cell suspension, the brain capillary-enriched fraction was incubated with 0.5 mg/ml Liberase Blendzyme Type I (Roche, Indianapolis, IN) and 120 U/ml DNase (Sigma Chemicals, St. Louis, MO) in PBS at 37°C for 30 min with agitation. DNase treatment reduces the viscosity of genomic DNA from damaged cells. The digested product was filtered through a 40-μm cell strainer (BD Biosciences, Franklin Lakes, NJ) and washed with PBS twice. The cells were resuspended in labeling buffer (0.5% (w/v) bovine serum albumin and 2 mM EDTA in PBS) and incubated with rat normal IgG (10 μg/10^7 cells, Chemicon, Temecula, CA, USA) as blocking reagent for 30 min at 4°C. Phycoerythrin (PE)-conjugated anti-rat CD31 antibody (0.4 μg/10^7cells, BD Bioscience) was added to the cell suspension and incubated for 30 min at 4°C. The cells were then washed with labeling buffer by centrifugation (430 g, 10 min, 4°C) and incubated with anti-PE Microbeads (20 μl/10^7cells; Miltenyl Biotec, Bergisch Gladbach, Germany), magnetic particles, for 15 min at 4°C. After washing the cells by centrifugation, they were resuspended in 500 μl labeling buffer. The cell sorting was performed by autoMACS (Miltenyl Biotec) according to the manufacturer's protocol and, then, the BCEC positively labeled with the magnetic microbeads were separated to obtain the positive fraction. The labeled cells were analyzed by flow cytometry using FACScalibur (BD Biosciences). Since the number of isolated cells is limited, viable cells were counted using the trypan blue exclusion test.

Quantitative Real-time PCR Analysis

Total RNA was extracted from rat whole brain, brain capillary fraction and MACS fractions with TRIzol Reagent (Invitrogen, Carlsbad, CA) and RNeasy kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Single-stranded cDNA was prepared from 1.2 μg total RNA by RT (ReverTraAce, Toyobo, Osaka, Japan) using oligo (dT) primer. Quantitative real-time PCR analysis was performed using an ABI PRISM 7700 sequence detector system (PE Applied Biosystems, Foster City, CA) with 2 X SYBR Green PCR Master Mix (PE Applied Biosystems) as per the

Table I. Primer Sets for RT-PCR

Gene	Accession No.	Forward Primer	Reverse Primer	Size (bp)
β-actin	NM_031144	TTT GAG ACC TTC AAC ACC CC	ATA GCT CTT CTC CAG GGA GG	352
PECAM-1	U77697	CTT CAC CAT CCA GAA GGA AGA GAC	CAC TGG TAT TCC ATG TCT CTG GTG	360
Claudin-5	BC002016	GCA GAG CAC CGG GCA CAT GC	TAG TTC TTC TTG TCG TAA TCG C	446
Tie-2	NM_013690	GGG CAA AAA TGA AGA CCA GCA C	GCA TCC ATC CGT AAC CCA TCC T	516
MAP-2	NM_013066	GGG TTT AAC TTT GGC CGG GGC CAT	CCT CTC CCA TCG CCA GTT CAA G	511
GFAP	NM_017009	TCC AAC CTC CAG ATC CGA GAA AC CA	TCC TTG TGC TCC TGC TTC GAC TCC	136
PDGFRβ	AY090783	TGG GCC AGA GTT CGT CCT CA	TCG GAG TCC ACT TCC CTG TC	492
α-SMA	X06801	GTA TTG TGC TGG ACT CTG GAG ATG G	GGA ATA GCC ACG CTC AGT CAG G	149
Desmin	BC061872	CTC AAG GCC AAG CTA CA	GTG ACT CAA TTC TGC GCT CTA	127
CD11b	AF268593	CCA GTG GTG AGA GTT CTA TC	CCC AGG TTG TTG AAC TGG TA	94
CD45	AH002194	ACA GGA GCT GGA GGA CAC AGC ACA	TTC GTC GGA TTC AGC CTC GCT CTC	149
CNPase	M18630	CCA AAT TCT GTG ACT ACG GGA AGG	AGG GCA GAG ATG GAC AGT TTG AAG G	106
Transthyretin	NM_012681	AAG ATC TTG CCA AAG CAG TAG C	AAT AAG AAC GTT TCA CGG CA	101
mdr1a	AY582535	ACA GAA ACA GAG GAT CGC	CGT CTT GAT CAT GTG GCC	437
Mrp1	AY170916	TGA ACC ATG AGT GTG CAG AAG GT	TCA CAC CAA GCC AGC ATC CTT	341
Mrp2	X96393	TGT TCT GTC CAA CGC CCT CAA	TCA CTT ATT CGC TCA ACT GCC ACA A	122
Mrp3	AF072816	GCC AAA GAT CGC AGG TGA GGA	AAG CAG GCA CCC ATG AGC AA	120
Mrp4	AY533524	AGA TAG TCA ACC TGC TGT CCA ACG A	GAG ATT CCG ATT TCC ACC CAG AGA	130
Mrp5	NM_053924	CCG GAA GAG GAA GAA GGC AAG CA	CCC ACA CTG CCA CAG ATT CCA ACC A	119
Mrp6	NM_031013	TGC ACG GGA TCA ACC TCA CC	CCA CGG AAC CCT CAA TGC TCA C	144

manufacturer's protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using pGEM-T Easy vector (Promega, Madison, WI) containing the corresponding gene fragments (dilution ranging from 0.01 fg/μl to 1 ng/μl). The fragment of all genes shown in Table I was amplified from total RNA prepared from rat whole brain, and each nucleotide sequence was confirmed using a DNA sequencer (CEQ2000XL, Beckman Coulter, Fullerton, CA). The control lacking the RT enzyme was assayed in parallel to monitor any possible genomic contamination. The PCR was performed through 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s after preincubation at 95°C for 10 min using specific primers. The sequences of the primers are indicated in Table I. After the PCR reaction, a single amplified product of the expected size was confirmed by agarose electrophoresis.

RESULTS

Purification of Rat BCECs by Magnetic Cell Sorting

The brief procedure for the purification of rat BCECs is shown in Fig. 1. The cells were treated with PE-conjugated anti-PECAM-1-antibody, and then reacted with anti-PE magnetic beads. The cells captured by magnetic column were isolated in the PECAM-1(+) fraction and the cells in flow through were collected as the PECAM-1(−) fraction. To optimize the recovery and purity of the BCECs, the type of enzyme, treatment time and concentration to disperse BCECs were examined. As far as the type of enzyme was concerned, Liberase Blendzyme Type I exhibited better cell dispersion than collagenase type I and type IV under microscopic observation. The concentration of Liberase Blendzyme Type I was tested at 0.1, 0.5 and 1.0 mg/ml, and the incubation time for 30, 60 and 90 min. Finally, the optimal conditions for purity and cell number were determined as for the brain capillary-enriched fraction treated with 0.5 mg/ml Liberase Blendzyme Type I for 30 min at 37°C for cell sorting. From five rats, $2.3 \times 10^6 \pm 0.2 \times 10^6$ cells were isolated in the PECAM-1(+) fraction (the mean ± SEM, $n = 6$). The viability of the cells in the PECAM-1(+) fraction was 86.2 ± 0.6 % (the mean ± SEM, $n = 4$). After cell isolation, the anti-PECAM-1-antibody labeled cells were analyzed by flow cytometry with the florescence intensity of PE as shown in Fig. 2. The percentage of positive cells was 85.9% ± 0.6 % and 4.8% ± 0.4 % in the PECAM-1(+) fraction and PECAM-1(−) fraction, respectively (the mean ± SEM, $n = 6$; Fig. 2a, b). When the cells were analyzed before reacting with antibody, no significant positive signal was detected (Fig. 2c).

Concentration of BCEC Marker mRNA in the PECAM-1(+) Fraction

To examine whether BCECs were concentrated in the PECAM-1(+) fraction, the mRNA levels of PECAM-1, claudin-5 and Tie-2, which are selectively expressed in BCECs in the brain (15–17), were measured as shown in Table II. The expression of PECAM-1, claudin-5 and Tie-2 mRNA in the PECAM-1(+) fraction was 35.7, 36.5 and 23.3-fold greater than that in the brain, respectively. Furthermore, the mRNA levels of each gene in the PECAM-1(+) fraction were also significantly greater than in the PECAM-1(−) fraction.

Less Contamination of Neural Cells in the PECAM-1(+) Fraction

To evaluate contamination of neural cells, the mRNA levels of various marker genes were compared between the PECAM-1(+) fraction and the brain capillary fraction isolated by the standard glass bead column method in Table III. The mRNA level of MAP-2, a neuron marker, was below the detection limit in the PECAM-1(+) fraction, whereas it was detected in the brain capillary fraction. As far as estimating the detection limit was concerned, the contamination of neurons in the PECAM-1(+) fraction was less than 3.1% of

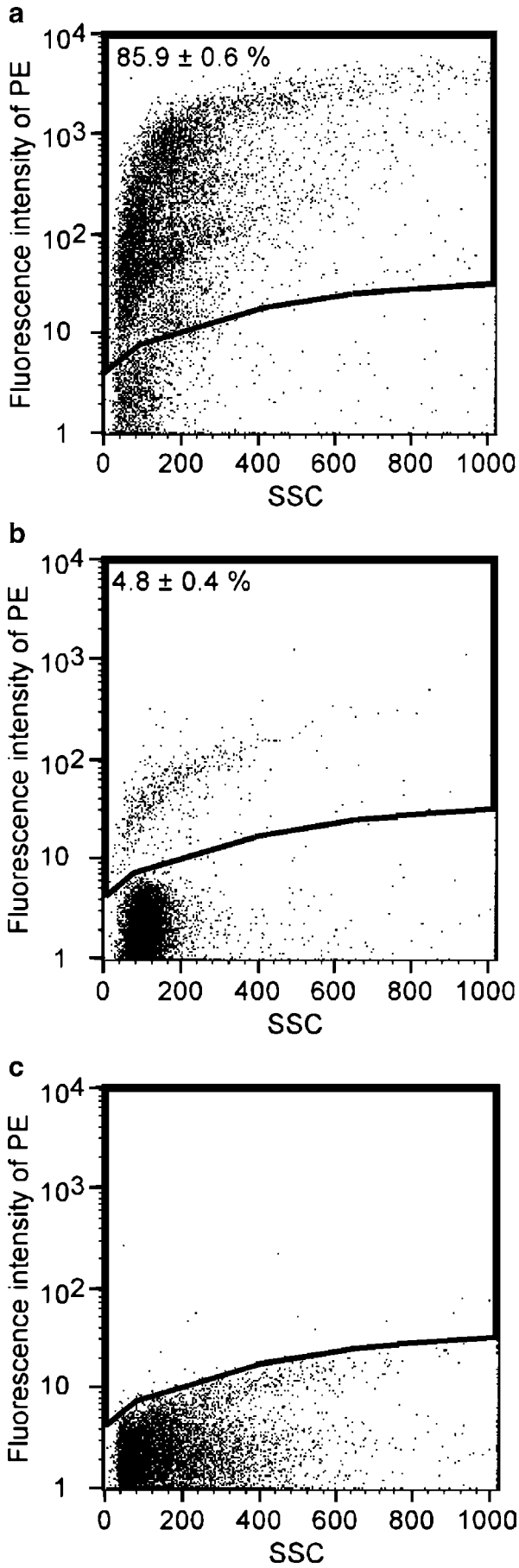

Table II. Expression Levels of Brain Capillary Endothelial Marker mRNA in Brain, and in PECAM-1(+) and (−) Fractions

	Target mRNA Levels ($\times 10^{-2}$ Target/β-Actin)		
	Brain	PECAM-1(+) fr.	PECAM-1(−) fr.
PECAM-1	0.975 ± 0.025	34.8 ± 1.5[a,b]	4.77 ± 0.45[a]
Claudin-5	8.46 ± 1.11	309 ± 25[a,b]	87.5 ± 8.6[a]
Tie-2	0.219 ± 0.019	5.11 ± 0.46[a,b]	1.43 ± 0.12[a]

The expression levels of target mRNA were normalized with respect to that of β-actin. Values represent the mean ± SEM of three separate preparations ($n = 6$)
[a] $p < 0.01$ significantly different from mRNA level in the brain
[b] $p < 0.01$ significantly different from mRNA level in PECAM-1(−) fraction

that in the brain capillary fraction. In the case of GFAP, an astrocyte marker, the mRNA levels in the PECAM-1(+) fraction was 0.093% of that in the brain capillary fraction.

The mRNA levels of all other marker genes shown in Table III were also lower or tended to be lower in the PECAM-1(+) fraction than in the brain capillary fraction, except for α-SMA, and all of these mRNA levels in the PECAM-1(+) fraction were also lower or tended to be lower than in the PECAM-1(−) fraction.

mRNA Expression Levels of Mrp Subtypes and Mdr1a in the PECAM-1(+) Fraction

mRNA levels of Mrp subtypes were examined using the same samples used in Tables II and III. As shown in Table IV, Mrp1 and 4 were predominantly expressed in the PECAM-1(+) fraction. Following these two subtypes, Mrp5 and 3 were abundantly expressed, whereas the mRNA levels were 10.6 and 7.60% of Mrp1 mRNA, respectively. The mRNA levels of Mdr1a in PECAM-1(+) was 1.21-fold greater than that of Mrp1.

The mRNA levels of Mrp3, 4 and 6 and Mdr1a were concentrated in the PECAM-1(+) fraction compared with the brain and PECAM-1(−) fraction. The mRNA level of Mrp2 in the PECAM-1(+) fraction was greater than that in the brain, but lower than that in the PECAM-1(−) fraction. The mRNA levels of Mrp1 and Mrp5 in the PECAM-1(+) fraction was 82.7 and 38.5% of that in the brain.

DISCUSSION

The present study initially demonstrated the establishment of a method to isolate highly purified fresh BCECs from rat brain. Isolation of vascular endothelial cells by using anti-PECAM-1 antibody and magnetic beads has been demonstrated from various tissues including brain (18–21). Demuele *et al.* (18) have reported the isolation of BCECs from rat brain, however, purity was assessed only by Mdr1a

◀**Fig. 2.** Flow cytometry analysis of the isolated fraction. Flow cytometry analysis of the PECAM-1(+) fraction (**a**), the PECAM-1(−) fraction (**b**) and cells before reacting with 1st antibody (**c**). *X* and *Y* axes indicate side scattered light (*SSC*) and fluorescence intensity of phycoerythrin (*PE*) detected by fluorescence channel 2 (*FL2*), respectively. The positive cells were counted in the gate indicated by the *thick line*.

Table III. Expression Levels of Non-BCECs Marker mRNA in Brain, Brain Capillary Fraction and PECAM-1(+) and (−) Fractions

		Target mRNA Levels ($\times 10^{-2}$ Target/β-Actin)			
	Marker for	Brain	Capillary fr.	PECAM-1(+) fr.	PECAM-1(−) fr.
MAP-2	Neurons	4.88 ± 0.28	0.417 ± 0.067	N.D.	N.D.
GFAP	Astrocytes	59.5 ± 7.1	64.9 ± 9.1	0.0601 ± 0.0141[b,c]	0.383 ± 0.110
PDGFRβ	Pericytes	0.222 ± 0.029	1.67 ± 0.22	0.366 ± 0.036[b]	0.491 ± 0.087
Desmin	Smooth muscle cells	0.853 ± 0.104	24.9 ± 3.2	15.9 ± 1.6[a,d]	37.2 ± 3.5
α-SMA	Smooth muscle cells	0.596 ± 0.064	17.2 ± 2.6	26.0 ± 3.1[d]	50.9 ± 7.0
CD11b	Microglia	0.0955 ± 0.0070	0.0787 ± 0.0117	0.0563 ± 0.0236[c]	0.179 ± 0.023
CD45	Leukocytes	0.0145 ± 0.0018	0.0768 ± 0.0117	0.0405 ± 0.0062[b,d]	0.338 ± 0.039
CNPase	Oligodendrocytes	22.8 ± 1.3	1.84 ± 0.14	0.420 ± 0.032[b]	0.443 ± 0.029
Transthyretin	Chroid plexus epithelial cells	436 ± 16	1500 ± 280	312 ± 49[b,d]	13400 ± 2000

The expression levels of target mRNA were normalized with respect to that of β-actin. Values represent the mean ± SEM of three separate preparations ($n = 6$). *N.D.*, not detected because below the detection limit
[a] $p < 0.05$, [b] $p < 0.01$ significantly different from the mRNA level in the brain capillary fraction
[c] $p < 0.05$, [d] $p < 0.01$ significantly different from the mRNA level in the PECAM-1(−) fraction

and GFAP expression. Wu *et al.* (21) have purified human BCECs by using anti-PECAM-1 antibody and magnetic beads after primary culture. In the present study, BCECs were isolated without cultivation and, additionally, using automated magnetic cell sorting (autoMACS), which can prevents technical variations during the isolation procedure. The concentration of BCECs and contamination by other neural cells were investigated by using various markers. As a result, BCECs were found to be concentrated in the PECAM-1(+) fraction and contamination by other neural cells was less than that in the brain capillary fraction isolated by the glass bead column method (Tables II and III). In particular, the contamination by neurons and astrocytes was markedly reduced in the PECAM-1(+) fraction. Therefore, the PECAM-1(+) fraction is more suitable for investigating the expression of molecules in BCECs than the brain capillary fraction isolated by classical methods.

The mRNA levels of desmin and α-SMA, markers for smooth muscle cells and/or pericytes (22), was concentrated in the PECAM-1(+) fraction and brain capillary fraction at similar levels compared with that in the brain (Table III). This is likely to be due to the contamination by smooth muscle cells and pericytes attached to brain vessels. The contamination of these cells could be prevented by detaching them from endothelial cells by enzyme treatment. However, longer treatment and/or a higher concentration of Liberase Blenzyme Type I resulted in cell damage and lower recovery (data not shown). For this reason, we established the treatment conditions for dispersing cells as shown in Fig. 1 to obtain a sufficient number of cells to conduct RT-PCR.

From five rats, 2.3×10^6 cells were isolated in the PECAM-1(+) fraction and 7.2 μg total RNA was prepared from this fraction. Although the number of cells was not enough to prepare a plasma membrane fraction for Western blot analysis, the amount of total RNA from one preparation was enough to conduct quantitative RT-PCR for about 70 individual molecules. PCR has a higher detection specificity and sensitivity than antibody, and proteolytic digestion of surface proteins cannot be excluded by protease treatment. Furthermore, protein levels cannot be compared between different molecules by using antibody due to the difference in antibody affinity for antigen. Thus, the expression levels of Mrp subtypes were measured in mRNA rather than protein in the present study, although the mRNA levels do not always correlate with the functional levels.

As shown in Table IV, Mrp1 and 4 were predominantly expressed in the PECAM-1(+) fraction and these expression levels were similar to that of Mdr1a. This result suggests that Mrp1 and 4 play key roles in the BBB transport system rather than other Mrp subtypes.

The expression profile of each Mrp subtype also suggests their localization in the brain. The mRNA levels of Mrp3, 4 and 6 were concentrated in the PECAM-1(+) fraction compared with the brain and PECAM-1(−) fractions (Table IV), suggesting that these subtypes are selectively localized in BCECs in rat brain. On the other hand, since the profile was different from that of BCEC markers as shown in Tables II and IV, the expression of Mrp1, 2 and 5 is not limited to BCECs in rat brain. In particular, the mRNA levels of Mrp1 were similar in the PECAM-1(+) fraction, the PECAM-1(−) fraction and the brain. Although contamination by astrocytes and neurons in the PECAM-1(+) fraction was almost

Table IV. mRNA Levels of Mrp and Mdr1a in Brain, and in PECAM-1(+) and (−) Fractions

	Target mRNA levels ($\times 10^{-3}$ Target/β-actin)		
	Brain	PECAM-1(+) fr.	PECAM-1(−) fr.
Mrp1	46.8 ± 4.7	38.7 ± 2.2	43.5 ± 5.4
Mrp2	N.D.	0.142 ± 0.034	0.239 ± 0.115
Mrp3	0.0755 ± 0.0253	2.94 ± 0.91[a]	N.D.
Mrp4	1.98 ± 0.39	25.0 ± 4.8[a,b]	8.19 ± 1.33[a]
Mrp5	10.7 ± 0.7	4.12 ± 0.45[a,b]	1.23 ± 0.13[a]
Mrp6	0.0470 ± 0.0064	0.493 ± 0.077[a,b]	0.120 ± 0.017[a]
Mdr1a	3.30 ± 0.35	46.7 ± 4.6[a,b]	11.7 ± 1.6[a]

The expression levels of target mRNA were normalized with respect to that of β-actin. Values represent the mean ± SEM of three separate preparations ($n = 6$)
N.D., not detected because below the detection limit
[a] $p < 0.01$ significantly different from the mRNA level in the brain
[b] $p < 0.01$ significantly different from the mRNA level in the PECAM-1(−) fraction

negligible (Table III), the possibility that the mRNA levels of Mrp1 in the PECAM-1(+) fraction included its mRNA expression in other neural cells could not be ruled out entirely. The predominant mRNA expression of Mrp1 in BCECs among Mrp1, 2 and 3 have been also reported in primary cultured bovine BCECs and isolated bovine capillaries (2). On the other hand, Mrp1 mRNA expression was not detected in primary cultured human and porcine BCECs by RT-PCR (9, 10). The latter studies compared Mrp1 mRNA expression during cultivation, and did not compare the mRNA levels between subtypes. Thus, a lack of mRNA detection by RT-PCR in these studies did not exclude the expression of Mrp1 in BCECs. Equally, there remains the possibility that Mrp1 expression in BCECs exhibits species differences.

Zhang *et al.* (2) have reported that the mRNA level of Mrp5 was greater than that of Mrp1 and 4 in the bovine brain capillary-enriched fraction. In this report, the brain capillary-enriched fraction was isolated by capillary depletion and there was no further purification of this fraction by the glass bead column method. The fraction prepared by capillary depletion was more contaminated than the fraction isolated by the glass bead column method. The present study demonstrated that Mrp5 was abundantly expressed in neural cells rather than BCECs (Table IV). Nies *et al.* (23) have also reported that, in human brain, MRP5 was detected not only in BCECs but also in neurons and astrocytes by immunohistochemical analysis using anti-human MRP5 antibody. Therefore, the highest level of Mrp5 mRNA in the bovine brain capillary-enriched fraction is likely to reflect the Mrp5 mRNA expression in contaminated neural cells rather than in BCECs.

ABCG2 is also expressed at the BBB, and is localized at luminal membrane of mouse, rat and human BCECs (24–26). Our previous report measured the mRNA expression levels of ABCG2 (11.6×10^{-3} target/β-actin) in the brain capillary fraction (25), and these were close to those of Mdr1a, Mrp1 and 4 shown in Table IV. Therefore, Mdr1a, ABCG2, and MRP1 and 4 appear to function as dominant efflux transporters cooperatively at the BBB. $E_2$17βG is eliminated from the brain by efflux transport at the BBB (27) and the involvement of Mrp1 in this efflux transport has been reported by using Mrp1-deficient mice (5). Since $E_2$17βG is a substrate of not only Mdr1a and Mrp1 but also ABCG2, and MRP 4 (1, 28, 29), the contribution of ABCG2 and Mrp4 to the BBB efflux transport of $E_2$17βG needs to be investigated. The BBB also carries out the efflux transport of dehydroepiandrosterone sulfate (DHEAS), and the involvement of the ABC transporter in this efflux has been suggested (30). DHEAS is a substrate of ABCG2, whereas its brain distribution is similar in ABCG2-deficient mice and wild-type mice (29, 31). DEHAS is also a substrate of Mrp4 (32). Therefore, Mrp4 is considered to contribute cooperatively or predominantly to the BBB efflux transport of DHEAS.

Mrp4 is the efflux transporter for nucleotide and nucleobase analogs, such as 6-mercaptopurine and azidothymidine monophosphate (33, 34). The BBB carries out the brain-to-blood efflux transport of 6-mercaptopurine and azidothymidine monophosphate, and restricts their brain distribution (35, 36). Such a low brain distribution may cause a significant reduction in the therapeutic effects on leukemia and viral infections. The present study suggests that Mrp4 is likely to be involved in the efflux transport of 6-mercaptopurine and azidothymidine monophosphate.

In conclusion, the present study demonstrated the purification of BCECs by magnetic cell sorting. The highly purified fresh BCECs isolated by the present method could be a useful material for the analysis of the expression of molecules at the BBB. Furthermore, Mrp1 and 4 is predominantly expressed in the isolated BCECs. For central nervous system-acting drugs, it will be necessary to develop compounds which are not recognized by not only Mdr1a/MDR1, but also ABCG2, Mrp1/MRP1 and Mrp4/MRP4. Since there still remains the possibility that the expression of Mrp subtypes in BCECs exhibits species differences, the expression levels of those efflux transporters at the human BBB requires future investigation in order to fully understand the brain distribution of drugs in humans.

ACKNOWLEDGEMENT

This study was supported, in part, by a Grant-in-Aid for Scientific Research on Priority Areas 17081002 from The Ministry of Education, Culture, Sports, Science and Technology of Japan and a 21st Century Center of Excellence (COE) Program grant from the Japan Society for the Promotion of Science.

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