Note

mRNA Expression and Amino Acid Transport Characteristics of Cultured Human Brain Microvascular Endothelial Cells (hBME)

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Summary: An in vitro cell culture system for estimating the human blood-brain barrier (BBB) permeability of drugs is required for the development of drugs with effects on the central nervous system. In this study, cultured human brain microvascular endothelial cells (hBME) were characterized. hBME cells exhibited concentration-dependent uptake of L-Leu, L-Glu and L-Lys with $K_m$ values of $51.1 \pm 23.1 \mu M$, $163.3 \pm 79.8 \mu M$ and $72.4 \pm 56.6 \mu M$, respectively. The cellular accumulation of rhodamine123 in hBME cells was unaffected by P-glycoprotein (P-gp) substrates (cyclosporin A, quinidine and verapamil), while the accumulation in human P-gp-overexpressing cells was significantly increased in the presence of these P-gp substrates. RT-PCR revealed that hBME cells expressed large neutral amino acid transporter 1 (LAT1) and its associated molecule (4F2hc), excitatory amino acid transporter 3 (EAAT3), cationic amino acid transporter 1 (CAT1), glucose transporter 1 (GLUT1), monocarboxylic acid transporter 1 (MCT1) and multidrug resistance-associated protein 1 (MRP1). However, no expression of multidrug resistance protein 1 (MDR1) was detected. The results suggest that these amino acid transporters are functionally expressed at the human BBB, and that hBME cells retain the in vivo BBB transport functions and expression characteristics. Consequently, hBME cells should be a useful tool for studies of the human BBB.

Key words: human brain microvascular endothelial cells (hBME); blood-brain barrier; amino acid transporters; multidrug resistance protein 1 (MDR1); multidrug resistance-associated protein 1 (MRP1)

Introduction

Cultured brain microvascular endothelial cells are an important in vitro model of the blood-brain barrier (BBB) and can be used to study the transport properties of the BBB. In particular, in studies to develop new drugs targeting the brain, this cell culture system can be expected to provide important information about the BBB permeability of compounds, thereby aiding the selection of potential drug candidates. This cell culture system could also be a useful tool for analyzing the physiological and biological functions of the BBB.

Primary cell cultures and immortalized cell lines of brain microvascular endothelial cells have been established and are widely used as in vitro BBB models. However, in most cases, these cultured cells have been derived from bovine, porcine, mouse and rat brain, and only rarely has human brain been used as a source. Estimating the BBB permeability of drugs at the human BBB on the basis of studies with non-human cultured cells is problematic, since marked species differences in transport at the BBB can be expected. In bovine, mouse and rat, P-glycoprotein (P-gp) is expressed at the luminal membrane of the BBB and is involved in the brain-to-blood efflux transport of drugs to limit their distribution into the brain. However, in humans, P-gp has been reported to be expressed at the foot processes of astrocytes, but not in the brain microvascular endotheli-
al cells. Therefore, cultured human brain microvascular endothelial cells could act as an in vitro human BBB model for the analysis of transport function and also to assist in drug development.

Human brain microvascular endothelial cells (hBME) are provided from the Applied Cell Biology Research Institute and have become commercially available from Dainippon Pharmaceutical. However, there has been no report about the gene expression of transporters or the transport function in hBME cells, and it is unclear whether these cells retain the in vivo BBB functions. Therefore, the purpose of the present study is to characterize the transport activity and to evaluate the expression of transporters in hBME cells as a potential in vitro human BBB model. Furthermore, our findings suggest that large neutral amino acid transporter 1 (LAT1), 4F2 heavy chain (4F2hc), excitatory amino acid transporter 3 (EAAT3) and cationic amino acid transporter 1 (CAT1) are expressed as amino acid transporters at the human BBB.

Materials and Methods

Materials: [14C]L-Leucine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [14C]Glutamic acid (258 mCi/mmol) and [14C]L-lysine (308 mCi/mmol) were purchased from NEN Life Sciences (Boston, MA, USA). Unlabeled L-Leu, L-Glu, L-Lys, rhodamine123, quinidine and verapamil were purchased from Sigma Chemicals (St. Louis, MO, USA). Cyclosporin A (CsA) was purchased from Wako Pure Chemicals (Osaka, Japan). All cell culture reagents were obtained from Gibco (Grand Island, NY, USA) and all other chemicals were commercial products of reagent grade.

Cell cultures: hBME cells provided by the Applied Cell Biology Research Institute (Kirkland, WA, USA) were purchased from Dainippon Pharmaceutical (Osaka, Japan). hBME cells were cultured in CS-C Complete Serum-Free medium kit (Cell Systems Corporation, Kirkland, WA, USA). hBME cells were grown at 37°C in a humidified atmosphere of 5% CO2 in air, and cells were passaged every four days. Cells at passage number 15–17 were used for this study.

Uptake study: hBME cells were seeded on 24-well culture plates (2.0 cm2, BIOCATTERN Collagen type I Cell ware 24-well plate, BD Biosciences, Billerica, MA, USA) at the density of 0.5 × 10⁶ cells/well. For [14C]L-Leu, [14C]L-Glu and [14C]L-Lys uptake measurements, hBME cells were used three days after seeding. Extracellular fluid buffer (ECF buffer: 122 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 0.4 mM K2HPO4, 10 mM HEPES and 10 mM D-glucose, pH 7.4, adjusted with 1 N NaOH) was used as the incubation medium. The cells were preincubated for 20 min at 37°C in 1 mL of incubation medium. The medium was replaced, and incubation was continued in 1 mL of incubation medium containing [14C]L-Leu (0.55 μCi), [14C]L-Glu (2.58 μCi) or [14C]L-Lys (3.08 μCi) at 37°C. The reaction was terminated by addition of 0.5 mL of ice-cold incubation solution followed by washing three times with 0.5 mL of ice-cold incubation solution. Then, the cells were solubilized with 0.1 N NaOH, and mixed with scintillation cocktail (Hionic-fluor, Packard, Meriden, CT, USA). The radioactivity in each sample was assayed in a liquid scintillation counter (LS6000TA, Beckman, Fullerton, CA, USA). The protein content of each sample was measured by the Lowry method with bovine serum albumin as a standard.

Accumulation study: hBME cells and LLC-COL150 cells were seeded on 24-well culture plates at densities of 0.5 × 10⁶ cells/well and 1 × 10⁶ cells/well, respectively. For the rhodamine123 accumulation studies, hBME cells and LLC-COL150 cells were used three days after seeding. ECF buffer (pH 7.4) containing 1% DMSO was used as the incubation medium. The cells were preincubated with 1 mL of incubation medium in the absence or presence of 10 μM test compound (CsA, quinidine and verapamil were used as P-gp substrates; L-Leu is not a P-gp substrate) for 15 min at 37°C. After preincubation, the medium was removed, substrate (10 μM rhodamine123 containing a test compound) was added to each well and incubation was continued for 45 min at 37°C. Then, the reaction was terminated by addition of ice-cold ECF buffer (pH 7.4) followed by washing three times with ice-cold ECF buffer (pH 7.4). The cells were then solubilized with 500 μL of 0.1 N NaOH and the concentration of rhodamine123 was determined by fluorescence spectrophotometry (emission at 485 nm/ excitation at 535 nm). The protein content of each sample was determined by the Lowry method.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis: Total RNA was isolated from hBME cells using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s instructions. Human brain total RNA was purchased from Clontech (Palo Alto, CA, USA). The RNA was reverse-
transcribed using oligo(dT) primer and ReverTra Ace (Toyobo, Osaka, Japan). For amplification, primers were designed as follows: MDR1, 5'-CCCCTCAT-TGCAATAGCAGG-3' (3017–3036) and 5'-GGCTGGGAGTTGA- 
TTGGAGCT-3' (67–90) and 5'-GGCCCGATT-GTCGCGATGAGGGCT-3' (611–588); glucose trans-
porter 1 (GLUT1), 5'-CCATTGACCTCTCTGTGGG- 
AACTTTGCTCATGAGGGCT-3' (1270–1289) and 5'-GGGACCTCA-
CTTCTCTGGTATTGGA-3' (1622–1603); CAT1, 5'-AGG- 
GACAAATTCTGGTGGATT-3' (1270–1289) and 5'-GGGACCTCA-
CTTCTCTGGTATTGGA-3' (1622–1603); CAT1, 5'-AGG- 
GACAAATTCTGGTGGATT-3' (1270–1289) and 5'-GGGACCTCA-
CTTCTCTGGTATTGGA-3' (1622–1603); CAT1, 5'-AGG- 
Algorithm: V = Vmax * C / (Km + C) + Kd * C
where V, C and Kd are the uptake rate, substrate concentration and non-saturable uptake clearance, respectively. Data analyses were performed using WinNonlin computer software, version 3.1 (Pharsight, Mountain View, CA, USA).

Results

Amino acid transport in hBME cells: hBME cells proliferated at 37°C with a doubling time of about 30 h, and appeared to have a spindle-fiber shaped morphology as shown in Fig. 1. hBME cells exhibited [14C]L-Leu uptake, which increased linearly up to 5 min before reaching a steady-state (Fig. 2). The initial uptake rate of [14C]L-Leu by hBME cells was found to be 15.8 ± 2.6 μL/(min·mg protein). The following analysis was performed after an incubation period of 2 min, and therefore reflects the initial uptake phase. The L-Leu uptake by hBME cells was concentration-dependent and composed of saturable and non-saturable components (Fig. 3A). The apparent Km and Vmax values of the saturable component were found to be 51.1 ± 23.1 μM and 2.4 ± 0.6 nmol/(min·mg protein), respectively. The uptake clearance (Kd) of the non-saturable component was 4.6 ± 0.7 μL/(min·mg protein).
Fig. 3. Concentration-dependence of L-Leu (A), L-Glu (B) and L-Lys (C) uptake by hBME cells. hBME cells were incubated for 2 min at 37°C with uptake buffer medium (pH 7.4) in the presence or absence of unlabeled L-Leu, L-Glu or L-Lys. The solid line in each figure was generated from equation (1) with the best fit parameters obtained by nonlinear least-squares fitting based on the Michaelis-Menten equation. Each point represents the mean ± S.E.M. (n = 3).

hBME cells exhibited uptake of L-Glu, an acidic amino acid, and L-Lys, a basic amino acid (Figs. 3B and C). These uptakes by hBME cells were also concentration-dependent, and the $K_m$ and $V_{max}$ values of the saturable component of L-Glu and L-Lys uptakes were found to be 163.3 ± 79.8 μM and 1.5 ± 0.5 nmol/(min·mg protein), respectively. The values of uptake clearance ($K_d$) of the non-saturable component of L-Glu and L-Lys uptake were 0.3 ± 0.4 μL/(min·mg protein) and 5.0 ± 0.8 μL/(min·mg protein), respectively.

Inhibitory effect of P-gp substrates on rhodamine123 efflux by hBME cells and LLC-COL150 cells: The uptake of 10 μM rhodamine123 by hBME cells and LLC-COL150 cells, which overexpress human P-gp (MDR1), was examined in the absence or presence of 10 μM P-gp substrates (CsA, quinidine and verapamil), and a non-P-gp substrate (L-Leu). In LLC-COL150 cells, rhodamine123 accumulated to the extent of 21.7 ± 1.2 μL/mg protein after a 45 min incubation period. CsA, quinidine and verapamil significantly increased the cellular accumulation of rhodamine123 by 419 ± 9%, 275 ± 3% and 350 ± 30%, respectively, compared with that of rhodamine123 alone, whereas L-Leu did not affect the accumulation of rhodamine123 (97.4 ± 13.2%). In hBME cells, rhodamine123 accumulated to the extent of 142 ± 7 μL/mg protein after a 45 min incubation period. In the presence of CsA, quinidine, verapamil and L-Leu, the relative values of cellular accumulation were not significantly changed, being 95.5 ± 1.2%, 89.6 ± 1.7%, 109 ± 5% and 102 ± 2%, respectively.

RT-PCR analysis of transporter gene expression in hBME cells: The mRNA expression of the molecules mediating transport at the BBB was examined by RT-PCR analysis using a specific primer set for each human transporter (Fig. 4). LAT1 and its associated molecule (4F2hc), EAAT3, CAT1, GLUT1 and MCT1, which are present in human brain, were detected in hBME cells. The products were of the expected size, and the nucleotide sequence of each product was identical with that of the corresponding human transporter. Regarding the mRNA expression of ATP binding cassette (ABC) transporters, MDR1 was not detected in hBME cells, though the gene product was amplified in human brain. The nucleotide sequence of the product from human brain was identical to that of MDR1. In contrast, MRP1 was detected in hBME cells, and the nucleotide sequence of the amplified product from hBME cells was identical to that of MRP1.

Discussion

The present study is the first to report the transporter expression and properties in commercially available hBME cells, which are used as an in vitro human BBB model. hBME cells exhibited uptake of acidic, basic and neutral amino acids and expressed transporters for amino acids, glucose and monocarboxylates. They also expressed MRP1 as an ABC transporter, but not MDR1. Primary culture cells and immortalized cells of brain microvascular endothelium have been reported to exhibit spindle-fiber morphology. The hBME cells also
exhibited spindle-fiber morphology, indicating that they retain the same morphology as the endothelial cells.

Transport of amino acids at the BBB plays a critical role in the overall regulation of several pathways of brain amino acid metabolism, including protein synthesis and neurotransmitter production. System L is an Na⁺-independent large neutral amino acid transport system, which transports bulky neutral amino acids, such as L-Leu, L-Val, L-Ile, L-Phe, L-Tyr and L-Trp. The presence of system L at the human BBB was confirmed using isolated human brain microvessels. In the present study, hBME cells exhibited [³H]L-Leu uptake in a time- and concentration-dependent manner, as shown in Figs. 2 and 3A, suggesting that hBME cells have a carrier-mediated L-Leu transport system closely similar to system L at the human BBB. The large neutral amino acid transporter is composed of a heterodimer of the 4F2hc heavy chain and the LAT1 light chain, and LAT1 has been reported to be selectively expressed at the BBB in bovine brain. hBME cells express both 4F2hc and LAT1 (Fig. 4), and the Kₘ value of L-Leu uptake by hBME cells (51.1 ± 23.1 μM; Fig. 3A) is similar to that by LAT1 and 4F2hc expressed in oocytes (19.7 μM). These results suggest that hBME cells retains system L that is present in human BBB in vivo, and LAT1 and 4F2hc are the molecules responsible for system L activity at the human BBB.

In the case of acidic amino acids, we showed that L-Glu undergoes brain-to-blood efflux transport at the rat BBB. A study using isolated rat brain microvessels has revealed that L-Glu transport at the BBB involves a high-affinity, Na⁺-dependent transport system. The L-Glu transport system at the BBB is believed to play an important role in maintaining a low L-Glu level in the brain; this is critical because of the excitotoxicity of L-Glu at high concentrations. However, there is a lack of information about the L-Glu transport system at the human BBB. As shown in Fig. 3B, hBME cells exhibited concentration-dependent L-Glu uptake, suggesting that human BBB possesses an L-Glu transport system like that of the rat BBB. Among the acidic amino acid transporters, the excitatory amino acid transporter (EAAT) family is Na⁺-dependent, and EAAT1–3 has been reported to be expressed in bovine brain microvascular endothelial cells. hBME cells also express EAAT3, as shown in Fig. 4. Furthermore, the Kₘ value of L-Glu uptake by hBME cells was found to be 163.3 ± 79.8 μM (Fig. 3B), which is similar to that of EAAT3-expressing COS-7 cells (62 μM). Therefore, the EAAT family also appears to be involved in acidic amino acid transport at the human BBB.
System y\(^+\) is an Na\(^+\)-independent basic amino acid transport system which transports L-Lys and L-Arg, and operates at the BBB. The cationic amino acid transporter (CAT1) is responsible for system y\(^+\) activity, and its expression in brain microvascular endothelial cells is 40-fold greater than that by whole brain.\(^{21}\) hBME cells exhibit L-Lys uptake with a \(K_m\) of 72.4 ± 56.6 \(\mu\)M, and express CAT1 mRNA (Figs. 3C and 4). The \(K_m\) value of CAT1 expressed in Xenopus oocytes has been reported to be 73 \(\mu\)M\(^{22}\) and is similar to that of hBME cells (Fig. 3C). These results suggest that a CAT1-mediated basic amino acid transport system exists at the human BBB. Uptake of these amino acids supports the idea that cultured hBME cells retain the amino acid transport systems that is present at the BBB in vivo.

Supply of energy sources, such as glucose and lactate, from the circulating blood to the brain is one of the important functions of the BBB. GLUT1 is expressed at the human BBB as a glucose transporter,\(^{23}\) MCT1 has also been reported to be expressed at the rat BBB, and mediates lactate transport.\(^{24}\) hBME cells express GLUT1 and MCT1 (Fig. 4), suggesting that hBME cells also retain the expression of transporters supplying energy to the brain.

ABC transporters, such as MDR1 and MRP1, mediate brain-to-blood efflux transport coupled with ATP hydrolysis and operate to limit drug permeability to the brain. While hBME cells expressed MRP1, no MDR1 was detected by RT-PCR, as shown in Fig. 4. Mouse, rat and bovine homologues of human MDR1 have been shown to be functionally localized at the luminal membrane of brain microvascular endothelial cells.\(^{3,4,25}\) In contrast, Partridge and co-workers have reported that human P-gp is localized on astrocyte foot processes at the abluminal face of the human brain microvascular membrane, rather than the endothelial luminal membrane.\(^{5,6}\) Our results, shown in Fig. 4, support their immunohistochemical findings. The apparent lack of P-gp in hBME cells was also examined by means of a transport study. Rhodamine123 is a substrate of P-gp, and inhibition of P-gp efflux transport activity by P-gp substrates, such as CsA, quinidine and verapamil, increases the rhodamine123 content of cells, as shown in LLC-COL150 cells. We found that CsA, quinidine and verapamil had no effect on rhodamine123 accumulation in hBME cells, as was also the case for L-Leu, which is not transported by P-gp. These results suggest that P-gp is not functionally expressed in hBME cells. This also appears to reflect the properties of the human BBB. However, P-gp expression is changed under various conditions, so that suppression of P-gp expression in the cultured cells cannot be ruled out. Further analysis is needed.

Expression of MRP1 mRNA was detected in hBME cells (Fig. 4). MRP1 is functionally expressed in primary cultured bovine brain microvascular endothelial cell (BBMEC) monolayer, and has also been detected in isolated rat and bovine microvessels.\(^{26,27}\) On the other hand, it was not detected in human brain microvessels, and the expression of MRP increased in cells during in vitro culture.\(^{9}\) Therefore, it remains necessary to clarify the contribution made by MRP1 to human BBB function.

This study has shown that hBME cells retain a number of BBB transport functions and gene expression properties. The directionality of transport is one of the most important properties of the BBB. L-Leu and L-Lys undergo both brain-to-blood and blood-to-brain transport.\(^{21}\) In contrast, L-Glu undergoes brain-to-blood efflux transport and EAAT3 is localized at the abluminal membrane of capillary endothelial cells.\(^{19}\) However, it is still unknown whether hBME cells retain the polarity of transport activity seen in the BBB in vivo. To clarify these issues, further studies to compare apical-to-basal and basal-to-apical transport by hBME cells and to quantify the expression of transporters in hBME cells and human brain capillaries will be required.

In conclusion, hBME cells contain several amino acid transport systems, and the molecules responsible are LAT1, 4F2hc, EAAT3 and CAT1. The human BBB would also express these amino acid transporters. Furthermore, hBME cells express GLUT1, MCT1 and MRP1, but lack MDR1 expression and P-gp function. hBME cells appear to retain the transport properties of the in vivo human BBB and, consequently, are expected to be a useful in vitro model of the human BBB.

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References
Characterization of Human Brain Microvascular Endothelial Cells


