mRNA Expression of the ATP-Binding Cassette Transporter Subfamily A (ABCA) in Rat and Human Brain Capillary Endothelial Cells

Sumio OHTSUKI,^{a,b,c} Yuki WATANABE,^a Satoko HORI,^{a,b,c} Hiroya SUZUKI,^a Jiraganya BHONGSATIERN,^a Masachika FUJIYOSHI,^a Mayu KAMOI,^a Naoko KAMIYA,^{a,b,c} Hitomi TAKANAGA,^{a,b,c} and Tetsuya TERASAKI^{*a,b,c}

^aDepartment of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University;

^bNew Industry Creation Hatchery Center, Tohoku University; Aoba, Aramaki, Aoba-ku, Sendai 980–8578, Japan; and

^cCREST and SORST of the Japan Science and Technology Agency (JST); Japan.

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The ATP-binding cassette transporter subfamily A (ABCA) consists of the transporters mediating cholesterol release and regulated by cholesterol. As about 25% of total body cholesterol exists in the brain, sterol homeostasis is an important issue as far as central nervous system function is concerned. The purpose of this study was to clarify the mRNA expression of ABCA subtypes at the blood–brain barrier (BBB) using cultured rat and human brain capillary endothelial cells, TR-BBB and hBME cells, respectively. mRNA expression of ABCA1, 2, 3, 4, 5, 6, 7 and 8/9 was detected in TR-BBB cells. In the brain capillary-rich fraction, mRNA expression of ABCA1, 2, 3, 4, 5, 7 and 8/9 was detected. ABCA2 and 5 mRNA were also detected in hBME cells. These results demonstrate, for the first time, that ABCA subtypes are expressed at the rat and/or human BBB. The expression of ABCA subtypes at the BBB is likely to contribute to sterol homeostasis in the central nervous system.

Key words ATP-binding cassette transporter subfamily A (ABCA); blood–brain barrier (BBB); mRNA expression; brain capillary endothelial cells; TR-BBB; hBME

The ATP-binding cassette (ABC) transporter subfamily A (ABCA) belongs to the ABC transporter superfamily, which also includes multidrug resistance protein 1 (MDR1/ABCB1), multidrug resistance associated proteins (MRP/ABCC) and breast cancer resistance protein (BCRP/ABCG2). One of the unique characteristics of the ABCA transporters is their involvement in sterol transport. ABCA1 and 7 mediate Apo-AI-dependent cholesterol release from cells,^{1,2)} and the ABCA1 mutation is linked to genetic diseases, such as Tangier disease, associated with a high density lipoprotein deficiency.³⁾ The mRNA levels of ABCA2, 3 and 7 have been reported to be up-regulated by cholesterol,^{4–6)} also suggesting involvement of ABCA transporters in sterol transport processes.

Brain capillary endothelial cells (BCECs) form the blood–brain barrier (BBB) with tight junctions. BCECs regulate exchange between blood and brain by expressing various transporters, including MDR1/ABCB1, MRP/ABCC and BCRP/ABCG2.⁷⁾ Sterols, especially cholesterol, are important components of the central nervous system (CNS). Indeed, about 25% of total body cholesterol is in the brain.⁸⁾ It has been reported that primary cultured porcine BCECs express ABCA1, and are involved in the release of cholesterol from cells.⁹⁾ This report proposes the hypothesis that the BBB plays an important role in sterol homeostasis in the brain. Abnormalities in sterol homeostasis have been reported to be associated with a number of neurodegenerative diseases, including Alzheimer's disease.⁸⁾ Therefore, clarifying the expression of ABCA subtypes in BCECs could be an important contribution to our understanding of the function of the BBB and sterol homeostasis in the CNS.

Cultured BCECs are a useful tool for examining the expression and regulation of transporters at the BBB. We have established several lines of conditionally immortalized BCECs (TR-BBB) from a transgenic rat harboring tempera-

ture-sensitive SV40 large T-antigen gene.^{10,11)} TR-BBB cells express transporters including mdr1a/ABCB1, mrp1/ABCC1 and BCRP/ABCG2, and also retain the functions of the *in vivo* BBB.^{12,13)} hBME cells are cultured human brain microvascular endothelial cells, which also express BBB transporters, including MRP1, and retain amino acid transport activity.¹⁴⁾

The purpose of this study was, therefore, to investigate the expression of ABCA subtypes in rat and human BCECs by using TR-BBB and hBME cells.

MATERIALS AND METHODS

Animals Adult male Wistar rats, weighing 230 to 280 g, were purchased from Charles River Laboratories (Yokohama, Japan). All experiments were approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Cell Culture TR-BBB cells and hBME cells were cultured as described previously.^{10,14)} Briefly, TR-BBB11 and 13 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20 mM sodium bicarbonate, 15 µg/ml endothelial cell growth factor, 100 U/ml benzylpenicillin potassium, 100 µg/ml streptomycin sulfate and 10% fetal bovine serum (Moregate, Bulimba, Australia) onto collagen type I-coated tissue culture dishes (BD Biosciences, Franklin Lakes, NJ, U.S.A.) at 33 °C in a humidified atmosphere of 5% CO₂ in air.

hBME cells provided by the Applied Cell Biology Research Institute (Kirkland, WA, U.S.A.) were purchased from Dainippon Pharmaceutical (Osaka, Japan). hBME cells were cultured in CS-C Complete Serum-Free medium kit (Cell Systems Corporation, Kirkland, WA, U.S.A.). hBME cells were grown in collagen type I-coated tissue culture dishes

* To whom correspondence should be addressed. e-mail: terasaki@mail.pharm.tohoku.ac.jp

Table 1. Primer Sets Used for RT-PCR

	Sense primer sequence Antisense primer sequence	Annealing temperature Product size
A1-1	TGAATAATATCAATGATATCCTGAAGTCCG GGGTTGGACCCGTGTAATCGTACAAC	60 °C 1063 bp
A1-2	CGGTTGAAGGAGACCATGCGCATC CCCATTGGACCCCGATGCCTTGCT	60 °C 424 bp
A2	CCCGGAAGATTGGCCGTATCCTGG TTGAAGGACAGCTGGGCCCGC	60 °C 1124 bp
A3	GGCTTCTCTACTTCTTCACTTA GGAAGACCACCACCTCTCCA	60 °C 823 bp
A4-1	AGCGCTTCATCATCTTCAGCCA TGACTCCAGGCTCTGGATCAATG	55 °C 506 bp
A4-2	AGGTCACCCTACAGTGGATAGAAG TGTGGAATCAATCCCCAGGAAGGC	65 °C 362 bp
A5	GATGATTCACTGAAGTGTATGGGTTA ATCTTAAGTCCAGACACCATGAT	60 °C 718 bp
A6	CAGTTCAAGTTTGGGCCACCTGGG CTCAAACATAGGGGTGATAGCAACCT	60 °C 1046 bp
A7-1	AGTAGTGGCTCGGCCTCCATCCTG AGCTCTGCCTCATCCAGGTGGTG	55 °C 428 bp
A7-2	GCCATGACGTGCAAACCAACATG AGTGGAGAGGATCAGTGTCCGAC	55 °C 380 bp
A8/9-1	CTTCTAGATGAGCCGTCCACTGGG ATCAGAGAAGAGTACCGTTCTCTGCC	55 °C 329 bp
A8/9-2	AGGCGATCCGGGCCACTTTTAC TTGAGCGACTCCACCAGAGCCG	62 °C 214 bp

Primer sets A1-1, A4-1, A7-1 and A8/9-1 were used for RT-PCR analysis in TR-BBB cells, and primer sets A1-2, A4-2, A7-2 and A8/9-2 were used in rat brain capillary rich fraction.

(BD Biosciences) at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Brain Capillary-Rich Fraction Isolation of the rat brain capillary-rich fraction was performed as described previously.¹⁰ Briefly, cerebrum excised from rats was dissected into pieces, and homogenized in phosphate-buffered saline (PBS). Homogenate was added to the same volume of 32% dextran solution, and then centrifuged (4500×g, 20 min, 4 °C). The resulting pellets were washed in PBS to obtain the enriched capillary fraction. The mRNA level of ABCG2, which is selectively localized at BCECs in rat brain, in the fraction was enriched 34.7-fold compared with that in whole brain.¹⁵

RT-PCR Analysis Total RNA was prepared from TR-BBB cells, hBME cells and brain capillary-rich fraction using TRIzol reagent (Life Technologies, Grand Island, NY, U.S.A.) according to the manufacturer's protocol. Human brain cDNA was purchased from Clontech (Palo Alto, CA, U.S.A.). Single-stranded cDNA was made from 1 µg total RNA by reverse-transcription (ReverTraAce, Toyobo, Osaka, Japan) using oligo dT primer. Primer sets were designed from the regions in which the nucleotide sequence was conserved in humans and mice, since, at the time the RT-PCR analysis was conducted, the nucleotide sequence of the rat ABCA subtypes was not available. PCR was conducted with the specific primer sets (Table 1) through 1 cycle of 94 °C for 2 min, and 35 cycles of 94 °C for 30 s, annealing temperature (Table 1) for 1 min, and 72 °C for 1 min, then 72 °C for a further 10 min. The RT-PCR products were separated by elec-

Fig. 1. mRNA Expression of ABCA Subtypes in TR-BBB Cells and Rat Brain Capillary-Rich Fraction

(A) mRNA expression in TR-BBB11 (11) and TR-BBB13 (13) cells. RT-PCR analysis was performed with specific primer sets shown in Table 1. For ABCA1, 4, 7 and 8/9 detection, primer sets A1-1, A4-1, A7-1, A8/9-1 were used, respectively. (B) mRNA expression in rat brain capillary-rich fraction. For ABCA1, 4, 7 and 8/9 detection, primer sets A1-2, A4-2, A7-2, A8/9-2 were used, respectively. Reactions were performed against total RNA with (+) or without (-) reverse transcription (RT).

Fig. 2. mRNA Expression of ABCA2 and 5 in Human Brain and hBME Cells

RT-PCR analysis was performed with the specific primer sets shown in Table 1. Reactions were performed against total RNA with (+) or without (-) reverse transcription (RT).

trophoresis on agarose gel. All PCR products were subcloned and sequenced using a DNA sequencer (CEQ2000XL; Beckman Coulter, Fullerton, CA, U.S.A.).

RESULTS

mRNA expression was analyzed using 2 lines of TR-BBB cells, TR-BBB11 cells and TR-BBB13 cells (Fig. 1A). ABCA1, 2, 3, 4, 6, 7 and 8/9 were detected in both TR-BBB11 and TR-BBB13 cells. ABCA5 was detected in TR-BBB11 cells, but not in TR-BBB13 cells. The nucleotide sequences of the amplified products of ABCA1, 2 and 5 were identical to those of the corresponding rat genes. The sequences of the product of other subtypes had the highest homology with the corresponding mouse genes (ABCA3, 86%; ABCA4, 93%; ABCA6, 89%; ABCA7, 93%; ABCA8, 86%; ABCA9, 92%). This result indicates that the ABCA subtypes are expressed in TR-BBB cells.

The expression of ABCA subtypes at the BBB *in vivo* was examined in the rat brain capillary-rich fraction. mRNA expression of ABCA1, 2, 3, 4, 7 and 8/9 was detected (Fig. 1B). This suggests that these ABCA subtypes are expressed in rat BCECs. By using primer sets for ABCA6, multiple bands were detected at around the expected size (data not shown).

Expression in human BCECs was examined using hBME cells. Due to the limitation of the cDNA, RT-PCT was performed for one subtype in each ABCA1- and ABCA5-like

subgroups, namely ABCA2 and 5, respectively. The amplified products of ABCA2 and 5 in hBME cells were detected at the same size as in human brain (Fig. 2). The nucleotide sequence of each product was identical to that of the corresponding human subtype. This result suggests that ABCA2 and 5 are expressed in human BCECs.

DISCUSSION

The present study examined the mRNA expression of ABCA subtypes in cultured rat and human BCECs (TR-BBB cells and hBME cells, respectively) and the rat brain capillary-rich fraction. The brain capillary-rich fraction is useful for detecting expression in the BBB *in vivo*, however, contamination by neural cells could not be ruled out. Indeed, ABCA1 was detected in cultured glial cells and neurons,¹⁶⁾ and ABCA2 is expressed in oligodendrocytes.¹⁷⁾ Cultured BCECs are free of any contamination, whereas immortalization affects gene expression. To minimize the difference in the gene expression between the *in vivo* BBB and cultured BCECs, we conducted PCR analysis using cultured rat and human BCECs and rat brain capillary-rich fraction. Furthermore, two different lines of TR-BBB cells were used to examine the line effect of immortalization on transporter expression. Among the ABCA subfamily, ABCA1 has been reported to be expressed in cultured porcine BCECs.⁹⁾ This study suggests that not only ABCA1 but also other ABCA subtypes are expressed at the BBB.

ABCA4 has been identified as a gene, the mutation of which is linked to eye conditions, such as Startgardt's disease,¹⁸⁾ and it has been reported to be selectively expressed in the retina by Western blot analysis.¹⁹⁾ This study shows that ABCA4 is expressed in TR-BBB cells and the brain capillary-rich fraction (Fig. 1), suggesting that ABCA4 is expressed at the BBB. This is the first evidence to suggest that ABCA4 is expressed in tissues other than the retina. Since the volume of BCECs is only 0.1% of the total brain, the expression of ABCA4 could not be detected in the brain by Western blot analysis. There is a clinical report showing that neurological conditions, such as weakness of both arms and legs, and signs of demyelination at the cerebellum are found in a patients with Startgardt's disease.²⁰⁾ Furthermore, ABCA4 has been suggested to mediate retinal transport,²¹⁾ and retinoids play important roles in CNS function and development.²²⁾ Therefore, expression of ABCA4 in BCECs would explain the neurological symptoms in Startgardt's disease.

Expression of ABCA2, 3 and 7 has been reported to be induced by cholesterol.^{4–6)} The present study suggests that these subtypes are expressed at the BBB. Therefore, it is conceivable that the plasma and brain levels of cholesterol affect the expression of ABCA2, 3 and 7 at the BBB. Interestingly, ABCA2 has also been suggested to act as a drug resistance gene, since ABCA2 protein is highly expressed in human ovarian cancer cells resistant to estramustine.²³⁾ ABCA2 is also expressed in hBME cells (Fig. 2). Therefore, it is possible that the cholesterol level affects the drug efflux process at the BBB.

The ABCA subfamily consists of two subgroups: one is the ABCA1-like subgroup formed with ABCA1, 2, 3, 4 and 7, and the other is the ABCA5-like subgroup formed with

ABCA5, 6, 8 and 9.²⁴⁾ Genes of the ABCA5-like subgroup cluster on human chromosome 17.²⁴⁾ Compared with the ABCA1-like subgroup, information about the ABCA5-like group is limited. ABCA5 mRNA was detected in TR-BBB cells, rat brain capillary-rich fraction and hBME cells (Figs. 1, 2). mRNA expression of other transporters in the ABCA5-like subgroup was also detected in TR-BBB cells (Fig. 1A). These results suggest that transporters in the ABCA5-like subgroup play roles in the rat and/or human BBB. ABCA5 mRNA was not detected in TR-BBB13 cells, while it was detected in TR-BBB11 cells, rat brain capillary-rich fraction and hBME cells (Figs. 1, 2). One possible explanation is down-regulation during cell culture. It is also conceivable that ABCA5 expression in the BCECs is heterogeneous in the brain, and TR-BBB11 and 13 cells were derived from different brain regions.

Among the ABCA5-like subgroup, ABCA8 has been reported to play a role in transport.²⁵⁾ Unlike other ABC transporters, ABCA8 exhibited ATP-dependent uptake of organic anions, such as estrone sulfate, *para*-aminohippuric acid and estradiol- β -glucuronide, in a *Xenopus* oocyte expression system. This indicates the possibility that ABCA8 functions as a new anion transport system at the BBB. ABCA8 and 9 have a high degree of homology, and the present PCR study could not distinguish these transporters by the sequence of the PCR product, since rat subtypes have not been reported, and ABCA9 was reported²⁶⁾ after this PCR analysis was performed. Therefore, further PCR analysis is necessary using a primer set in the non-conserved region, *e.g.* the un-translated region, to distinguish between ABCA8 and 9.

In conclusion, the present study shows that various ABCA subtypes are expressed in cultured rat and human BCECs, suggesting the expression of ABCA subtypes at the BBB. This BBB expression of ABCA subtypes throws a new light on studies involving the BBB. Therefore, the function and localization of ABCA subtypes at the BBB needs to be analyzed in further studies to gain a better understanding of BBB function.

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