Functional Clarification of MCT1-Mediated Transport of Monocarboxylic Acids at the Blood-Brain Barrier Using *In Vitro* Cultured Cells and *In Vivo* BUI Studies

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Received July 14, 1999; accepted October 13, 1999

Purpose. To prove the functional significance of monocarboxylic acid transporter, MCT1 at the blood-brain barrier (BBB) for the passage of both endogenous and exogenous monocarboxylic acids into the central nervous system.

Methods. Monocarboxylic acid transport at the BBB was studied in rats by using a newly established immortalized brain capillary endothelial cell (BCEC) line, RBEC1, and the results were compared with those obtained by using primary cultured BCECs, cells stably expressed with rat MCT1, and the *in vivo* brain uptake index (BUI) method.

Results. The cell line, RBEC1 meets various morphological and enzymatic criteria of BCECs and appears to be suitable for the study of BBB transport of monocarboxylic acids. The presence of MCT1-transcript in RBEC1 was confirmed by the RT-PCR method, as previously observed in isolated brain capillaries. A typical substrate of MCT1, lactic acid, was taken up by RBEC1 in a stereospecific and saturable manner. The value of the kinetic parameter *Km* showed good agreement with values previously obtained in studies using an *in vivo* BUI and *in vitro* MCT1-transfected cells. An organic weak acid, benzoic acid, which has been considered to cross biological membranes by passive diffusion, exhibited carrier-mediated transport properties, such as saturation, pH dependence, and stereospecific inhibition in RBEC1, similar to those we observed in primary cultured rat BCECs. The *Km* values in RBEC1, in primary cultured BCECs and in the *in vivo* BUI method were comparable and well agreed with that obtained in MCT1-transfected cells, suggesting that the transport features of benzoic acid observed by *in vitro* methods well reflect the *in vivo* transport activity. Furthermore, hybrid depletion of MCT1 in RBEC1 using an antisense oligonucleotide against rat MCT1 abolished the saturable transport of benzoic acid.

Conclusions. These observations show that MCT1 plays a significant role in the transport of monocarboxylic acids, including the exogenous organic weak acid benzoic acid, as well as native lactic acid.

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ABBREVIATIONS: BBB, blood-brain barrier; BCEC, brain capillary endothelial cell; BUI, brain uptake index; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino) ethanesulfonic acid.

KEY WORDS: blood-brain barrier; monocarboxylic acid; MCT1; immortalized cell; benzoic acid; membrane transport.

INTRODUCTION

The BBB strictly restricts the penetration of endogenous and exogenous compounds into the brain, and is formed by brain capillary endothelial cells (BCECs) linked to each other by tight junctions, with little capacity for pinocytosis and few fenestrations. Because of these unique anatomical characteristics, the BBB is thought to comprise the major barrier to the entry of hydrophilic or high-molecular-weight compounds into the brain (1). This idea is also supported by the fact that the observed permeability of the BBB to drugs is usually well correlated with the lipophilicity of the drugs (2). On the other hand, the BBB is well known to have several influx and efflux transport systems that serve to maintain homeostasis of the brain parenchyma. Recently, it has been found that these transport mechanisms play important roles in the transfer of drugs between the bloodstream and the brain extracellular space across the BBB. For example, the apparently low permeability of the BBB to several cationic and neutral drugs such as cyclosporin A and vincristine was clarified to be due to the function of P-glycoprotein, which is a primary active efflux transporter located on the luminal membrane of cerebral capillary endothelial cells (3–5).

So far, cDNAs of seven members of the proton-coupled monocarboxylic acid transporter MCT family have been identified from mammals, and their cDNA sequences and tissue distributions have been summarized (6). The first MCT isoform, MCT1, was cloned from Chinese hamster ovary cells (7), and mediates the transport of lactic acid and pyruvic acid. We cloned rat MCT1 from small intestinal mucosal cells (8). Northern blot analysis showed that MCT1 is widely distributed in various tissues, including whole brain. Moreover, the MCT1 gene was detected in rat brain capillaries by using an RT-PCR method (8). Immunohistochemistry with MCT1-specific antibody showed that MCT1 is present at both luminal and abluminal membranes of the brain capillary endothelial cells (9). As lactic acid and ketone bodies are abundant in the brain (10,11), it is expected that MCT1 at the BBB may contribute to the regulation of energy substrates in the brain parenchyma.

Kinetic studies have indicated the presence of a short-chain fatty acid transport system at the BBB. Using the *in vivo* brain uptake index method (BUI), it was confirmed that several short-chain fatty acids are transported across the BBB (12). In particular, lactic acid transport showed stereospecificity and pH dependence (13). Furthermore, acetic acid and monocarboxylic acid drugs such as salicylic acid appeared to be transported by a monocarboxylic acid-specific transporter having characteristics similar to those of the lactic acid transporter, based on studies using *in vivo* BUI and *in vitro* primary cultured bovine BCECs (14,15). However, the transporters involved have not yet been fully identified at the molecular level. In addition, we have already shown that MCT1 is responsible for the transport of organic weak acids (8,16,17). However, the relevance of MCT1 to the BBB transport of exogenous organic acids remains to be clarified. Additionally, the usual criteria, such as saturation, pH-dependence and competitive inhibition, do not necessarily prove the existence of carrier-mediated transport in the uptake

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of highly membrane-permeable compounds, as argued on the basis of the studies using liposomes as artificial membranes (18). Therefore, molecular identification of the responsible transporter is essential to clarify the mechanism of the transport of organic weak acids at the BBB.

In the present study, we obtained functional and molecular evidence that MCT1 is responsible for the transport of exogenous as well as endogenous monocarboxylic acids at the BBB. To evaluate the MCT1 transport activity at the BBB by *in vitro* experimental methods and to compare the results directly with those of an *in vivo* study, we established immortalized rat BCECs that express rat MCT1, since rats are useful for *in vivo* BBB transport studies and cloned rat MCT1 cDNA is available. Furthermore, to demonstrate the importance of MCT1 for anionic drug permeation across the BBB into the brain, we used benzoic acid as a model of a monocarboxylic acid drug, which has been confirmed to be transported by rat MCT1 (17).

MATERIALS AND METHODS

Materials

[^{14}C]Benzoic acid (19.0 mCi/mmol), [^{14}C]sucrose (4.5 mCi/mmol) and [^{3}H]water (1.0 mCi/g) were purchased from DuPont NEN (Boston, MA USA). L-[^{14}C]Lactic acid (150 mCi/mmol) and [^{3}H]sucrose (12.0 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) and Amersham (Buckinghamshire, England), respectively. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and horse serum (HS) were obtained from GIBCO Chemical Industries Ltd. (Osaka, Japan), rat tail collagen (Type I) was from Collaborative Research Inc. (Grand Island, NY), collagenage/dispase and dispase were from Boehringer Mannheim GmbH (Mannheim, Germany), dextran (industrial grade, MW 87,000), gentamicin sulfate, heparin, endothelial cell growth factor (ECGF) and Percoll were from Sigma Chemical Co. (St. Louis, MO), and Ketaral 50 (ketamine hydrochloride) was from Sankyo Co., Ltd. (Tokyo, Japan). Mouse anti-rat transferrin receptor monoclonal antibody was from Chemicon International Inc. (Temecula, CA). All other chemicals were commercial products of reagent grade.

Isolation and Culture of Rat BCECs

The study was performed according to the Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University and was approved by the Committee of Ethics of Animal Experimentation of Kanazawa University, Takara-machi Campus. Rat primary BCECs were isolated from cerebrum of male Wistar rats (SLC, Shizuoka, Japan), 4–5 weeks old as described previously (19). The isolated BCECs were seeded at 50,000 cells/cm^2 on collagen-coated culture dishes (48-well cluster dish, diameter 12 mm) and cultured at 37°C in a 5% CO_2/95% air atmosphere. For the first three days of culture, the medium consisted of DMEM containing 20% FCS, 20 mM sodium bicarbonate, 2 mM L-glutamate, 50 IU/ml penicillin G, 50/μg/ml streptomycin, 2.5 μg/ml amphotericin B, 50 μg/ml ECGF and 10 U/ml heparin. Then, it was replaced with the same medium but with 5% FCS and 5% HS and without heparin or amphotericin B; thereafter, the medium was replaced with fresh medium every other day. Uptake experiments were performed when the cells reached confluence in approximately 10 to 12 days.

Establishment of Rat Brain Capillary Endothelial Cell (RBEC) Line

After isolation of BCECs from rat brain, primary cultured rat BCECs were immortalized by transfection of recombinant plasmids containing origin-defective SV40 gene, SVori-8-16 (20). Briefly, the cells were seeded into 35 mm collagen-coated dishes (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) with 2.5 ml of the first three days′ medium. After cultivation for 1 or 2 days, 200 μl of plasmid DNA mixture (30 μg/ml) was added to the dish and incubation was continued overnight at 37°C under 3% CO_2 as previously described (21). The medium was removed, and the cells were rinsed, and incubated at 37°C under 5% CO_2. Actively growing cells were inoculated into 96-well culture plates at low density and incubated in a CO_2 incubator. Twenty-one independent colonies were isolated about 2 weeks later to establish clonal cell lines. The isolated RBEC clones were cultured in the same way as the primary cultured BCECs. The clone designated RBEC1 was selected for the present study on the basis of several criteria, including enzyme expression, growth rate and apparent morphology. For the uptake study, RBEC1 was seeded at 50,000 cells/cm^2 on collagen-coated culture dishes (16 mm diameter).

As the marker for the BCECs, the expression of transferrin receptor was measured (22,23). Immunodetection of this protein was performed by using DAKO LSAB(R)2 KIT Peroxidase (Dako) and primary antibody (OX-26, Chemicon International Inc., Temecula, CA) as described. γ-Glutamyltranspeptidase activity was measured according to the procedure previously reported (24), using whole brain as the positive control. A unit of enzyme activity is defined as the amount of γ-glutamyltranspeptidase catalyzing formation of 1 nmol p-nitroaniline per min at 37°C. The cell monolayers were then washed 3 times with culture medium and examined on a microscope.

Detection of Rat MCT1 in RBEC1

Total RNA was isolated from RBEC1 by means of the standard method using acid guanidinium-phenol-chloroform. Five μg of total RNA was used as the template for the reverse transcriptase reaction and the resultant DNA was used for further PCR reaction under the conditions reported previously (8). The specificity of the primer pairs was established by PCR using cDNA of rat MCT1 as the template. The RT-PCR products were analyzed by agarose gel electrophoresis.

Uptake Experiments

Both primary cultured and immortalized rat BCECs were cultivated on multiwell dishes. When they reached confluence, they were washed three times with 1 ml of incubation solution containing 122 mM NaCl, 3 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 1.4 mM $CaCl_2$, 10 mM D-glucose and 10 mM Mes (pH 5.5, 6.0, 6.5) or Hepes (pH 7.0, 7.5) and preincubated at 37°C for 30 min. After the preincubation, test solution (0.25 ml) containing radiolabeled test compound was added to initiate uptake. The cells were incubated at 37°C for the designated time, then washed three times with 1 ml of ice-cold incubation solution to terminate the uptake.

For the quantitation of the radioactivity taken up, the cells were solubilized with 1 N NaOH for 120 min, then neutralized with HCl, and radioactivity was determined. Cellular protein content was measured by the method of Lowry *et al.* (25) using BSA as a standard. Net uptake was expressed as the uptake rate (nmol/30 sec/mg protein) or the cell-to-medium ratio (μl/mg protein) which was obtained by dividing the apparent accumulated amount per milligram of protein by the concentration of substrate in the incubation medium after correction for extracellularly adsorbed lactic acid and benzoic acid estimated from the apparent uptake of [^{3}H]sucrose, a membrane non-permeable marker. In order to estimate the kinetic parameters for the uptake by BCECs, the uptake rate was fitted to the following Eq. (1) by nonlinear least-squares regression analysis.

$$V/C = Vmax/(Km + C) + kd \quad (1)$$

Here, *Km* and *Vmax* are the Michaelis constant and the maximum uptake rate, respectively, for a carrier-mediated process, *kd* is the apparently nonsaturable first-order rate constant.

Hybrid Depletion Experiments

The following oligonucleotides were used in the present study: rat MCT1 sense oligonucleotide 5′-ATGCCACCTGCGATTGGCGGGCC-3′ (corresponding to bases 206–229 of rat MCT1 cDNA sequence) (8) and rat MCT1 antisense oligonucleotide 5′-GGCCCGCCAATCGCAGGTGGCAT-3′. RBEC1 cells were seeded into wells (0.25×10^5 cells/ml) and after the cells had become attached to the wells, oligonucleotides (final concentration of 5 μM) were transfected into them with Lipofectamine (Life Tecchnologies, Inc., Gaithersburg, MD) at 4 ng/ml for six hours. After three days, the RBEC1 cells were transfected with oligonucleotides again in the same way, and used for uptake assay, on the following day.

In Vivo Brain Transport Study

The *in vivo* BBB transport was evaluated by the brain BUI method (14). The injection solution contained both a test compound, [^{14}C]benzoic acid, and a reference compound, [^{3}H]water, in Ringer-Mes buffer (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 10 mM Mes, pH 6.0). Rats, 8 weeks old, were decapitated at five seconds after intra-carotid artery injection of about 200 μL of injection solution, and the radioactivity in the brain tissue was measured. Brain uptake rate of benzoic acid (V), obtained by using Eq. (2) to (4) from the BUI values of benzoic acid ($BUI_{benzoic\ acid}$) and [^{14}C]sucrose ($BUI_{sucrose}$), a nonpermeable vascular marker (3.03% at five seconds after injection), the extraction ratio of a reference compound (^3H_2O, $E_{water} = 0.86$), and the brain blood flow F (0.93 ml/min/g), was used to calculate unidirectional clearance (V/C′) as reported previously (26,27). Here, C′, the mean capillary concentration, was obtained from C, the concentration in the injected solution by using the following Eq. (3) as reported by Pardridge *et al.*, (28). Michaelis constant *Km*, maximum uptake rate *Vmax*, and apparently nonsaturable first-order rate constant *kd* values were calculated by nonlinear least-squares regression analysis using equation [5].

$$E_{benzoic\ acid} = (BUI_{benzoic\ acid} - BUI_{sucrose}) \times E_{water} \quad (2)$$

$$C' = E_{benzoic\ acid} \times C/(-\ln(1 - E_{benzoic\ acid})) \quad (3)$$

$$V = E_{benzoic\ acid} \times F \times C' \quad (4)$$

$$V/C' = Vmax/(Km + C') + kd \quad (5)$$

RESULTS

Characterization of RBEC1

RBEC1 was selected for the present study from among the established cell lines on the basis of various criteria of BCEC. The apparent morphology of RBEC1 showed spindle-shape (Fig. 1A). The γ-glutamyltranspeptidase activities in the brain homogenate and RBEC1 were 3.64 ± 0.08 and 4.70 ± 0.14 units/mg protein respectively (Fig. 1B). The enzyme activity in RBEC1 was 1.3 times higher than that in the brain homogenate. By immunodetection with anti-transferrin receptor antibody (OX-26), the expression of transferrin receptor antigens was detected in RBEC1 (Fig. 1C).

Detection of Rat MCT1 Transcript in RBEC1

The presence of rat monocarboxylate transporter, MCT1 in RBEC1 was examined by the RT-PCR method using specific primers based on the nucleotide sequence of rat MCT1 (Fig. 2). The PCR product obtained from total RNA of RBEC1 had a size of 520 bp, which was the expected size, as was confirmed in the control study using rat MCT1 cDNA as the template. The detection of rat MCT1 transcript in RBEC1 is consistent with the presence of MCT1 in isolated rat brain capillaries (8).

Uptake of L-[^{14}C]Lactic Acid by RBEC1

To examine whether RBEC1 has MCT1 transport activity, we measured the initial uptake of L-[^{14}C]lactic acid, a well-recognized substrate of MCT1. L-Lactic acid uptake by RBEC1 was saturable. At pH 6.0, *Km*, *Vmax* and *kd* were obtained as 2.72 ± 0.77 mM, 46.1 ± 8.71 nmol/30 sec/mg protein and 0.48 ± 0.30 μl/30 sec/mg protein, respectively (date not shown). The estimated *Km* value was very similar to that of rat MCT1 expressed in MDA-MB231 cells (2.28 ± 0.47 mM) (17). As shown in Table I, L-[^{14}C]lactic acid uptake at 4°C was reduced to 23% of the control. L-and D-lactic acid inhibited the uptake in a concentration dependence manner. These observed properties are suggesting the participation of a carrier-mediated transport mechanism. Further, L-lactic acid and (S)-mandelic acid exhibited significantly stronger inhibitory effects on L-[^{14}C]lactic acid uptake than did their stereoisomers, suggesting the involvement of a stereo-specific transport mechanism. Other monocarboxylic acids, benzoic acid and valproic acid, significantly reduced the uptake of L-[^{14}C]lactic acid, while a dicarboxylic acid, succinic acid, was not inhibitory. The observed stereospecificitiy, and monocarboxylic acid-specific inhibition of L-[^{14}C]lactic acid uptake are consistent with the known functional properties of rat MCT1 (8,17).

Comparison of [^{14}C]Benzoic Acid Uptake in Primary Cultured BCECs and Immortalized BCECs (RBEC1)

The concentration dependence of benzoic acid uptake by RBEC1 and by rat primary cultured BCECs was examined. Initial uptake of [^{14}C]benzoic acid by rat primary cultured BCECs (Fig. 3A) and RBEC1 (Fig. 3B) was saturable. The kinetic parameters

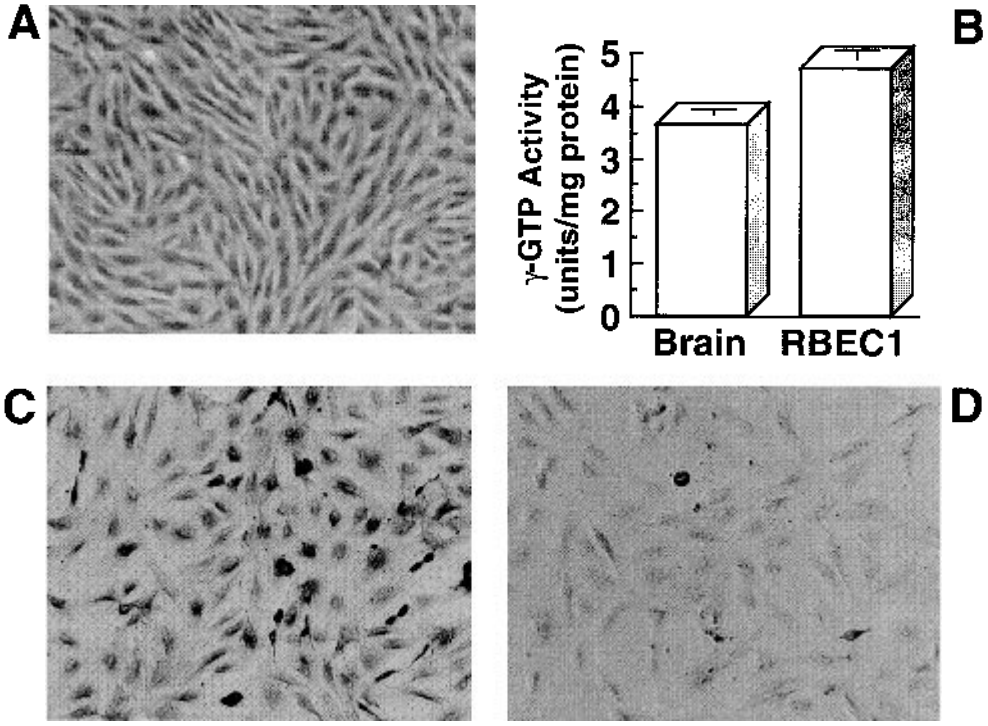

Fig. 1. Characterization of immortalized rat brain capillary endothelial cells (RBEC1). (A) Morphology of RBEC1. Phase contrast microscopy (4 × 15) showing the typical spindle shape morphology of cells forming a monolayer on collagen-coated dish. (B) γ-Glutamyl transpeptidase activities in RBEC1. Specific activities of γ-glutamyl transpeptidase are expressed as units/mg protein. Each point represents the mean ± S.E.M. of four experiments. Expression of antigens of transferrin receptor in RBEC1. (C) RBEC1 were stained with anti-transferrin receptor antibody (OX-26). (D) Normal mouse IgG were used as negative control.

in RBEC1 were *Km* of 1.15 ± 0.86 mM, *Vmax* of 12.2 ± 6.08 nmol/30 sec/mg protein and *kd* of 0.72 ± 0.59 μl/30 sec/mg protein, while those in rat primary cultured BCECs were *Km* of 2.89 ± 2.47 mM, *Vmax* of 18.6 ± 14.5 nmol/30 sec/mg protein and *kd* of 1.23 ± 0.95 μl/30 sec/mg protein, respectively. It appears that RBEC1 and rat primary cultured BCECs have similar affinity and capacity for benzoic acid transport.

Characterization of [^{14}C]Benzoic Acid Uptake by RBEC1

To elucidate the properties of the transporter responsible for benzoic acid uptake by RBEC1, the inhibitory effects of various compounds on [^{14}C]benzoic acid uptake were studied (Table II). All monocarboxylic acids, such as mandelic acid, acetic acid, propionic acid, butyric acid, salicylic acid and foscarnet (phosphonocarboxylic acid) significantly reduced [^{14}C]benzoic acid uptake. In contrast, a dicarboxylic acid, succinic acid, had no effect. L-Lactic acid exhibited significantly more potent inhibitory effects on [^{14}C]benzoic acid uptake than did D-lactic acid, Although no statistically significant difference on inhibitory effects between (S)-and (R)-mandelic acid was observed, (S)-mandelic acid showed tendency to inhibit the [^{14}C]benzoic acid uptake more strongly than did (R)-mandelic acid. These properties of [^{14}C]benzoic acid uptake were similar to those observed in L-[^{14}C]lactic acid uptake by RBEC1 (Table I).

Effect of pH Gradient on Benzoic Acid Uptake by RBEC1

The effect of a pH gradient on [^{14}C]benzoic acid uptake by RBEC1 was examined. The initial uptake of [^{14}C]benzoic acid increased with increasing H^+ concentration (Fig. 4). At a concentration of 0.1 mM [^{14}C]benzoic acid, uptake at pH 6.0 was almost three times faster than that at pH 7.4, whereas no significant pH-dependent transport was observed in the presence of an excess of unlabeled benzoic acid (10 mM).

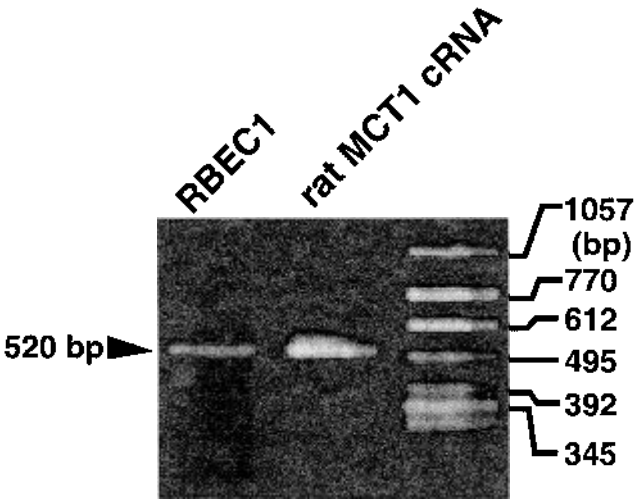

Fig. 2. Detection of MCT1 in RBEC1 by the RT-PCR Method. Total RNA from RBEC1 and rat MCT1 cRNA (control) were subjected to RT-PCR using primer pairs specific for rat MCT1 cDNA. The RT-PCR products were analyzed by agarose gel electrophoresis. The arrowhead shows the expected size of the PCR product corresponding to the PCR-primers used.

Table I. Inhibitory Effects of Various Compounds of L-[^{14}C]lactic Acid Uptake by RBEC1

Inhibitor	Concentration (mM)	Relative uptake (% of Control)
4° C		23.4 ± 3.61[a]
L-Lactic acid	2.5	70.3 ± 3.04[a]
	10	38.4 ± 0.77[a]
D-Lactic acid	2.5	91.0 ± 5.82[b]
	10	50.1 ± 4.14[a,b]
(S)-Mandelic acid	10	31.1 ± 1.72[a]
(R)-Mandelic acid	10	79.1 ± 1.96[a,b]
Benzoic acid	10	23.5 ± 0.91[a]
Valproic acid	10	38.6 ± 4.51[a]
Succinic acid	10	102.5 ± 0.54

[a] Significantly different from the control value ($p < 0.05$).
[b] Significantly different from the uptake value in the presence of stereoisomers at the same concentration ($p < 0.05$).
Note: Uptake of L-[^{14}C]lactic acid (100 μM) was measured at pH 6.0 37°C for 30 sec by incubating RBEC1 in the presence of each inhibitor. Each point represents the mean ± S.E.M. of 3 or 4 experiments.

Table II. Inhibitory Effects of Various Compounds on [^{14}C]Benzoic Acid Uptake by RBEC1

Inhibitor	Concentration (mM)	Relative uptake (% of Control)
L-Lactic acid	2.5	37.1 ± 1.40[a]
D-Lactic acid	2.5	59.1 ± 4.29[a,b]
(S)-Mandelic acid	2	40.8 ± 2.77[a]
(R)-Mandelic acid	2	57.3 ± 9.46[a]
Acetic acid	10	31.7 ± 4.21[a]
Propionic acid	10	54.4 ± 3.98[a]
Butyric acid	10	32.0 ± 3.21[a]
Salicylic acid	10	32.5 ± 3.72[a]
Foscarnet	10	45.8 ± 3.19[a]
Succinic acid	10	102.2 ± 12.8

[a] Significantly different from the control value ($p < 0.05$).
[b] Significantly different from the uptake value in the presence of L-isomers ($p < 0.05$).
Note: Uptake of [^{14}C]benzoic acid (100 μM) was measured at pH 6.0 and 37°C for 30 sec by incubating RBEC1 in the presence of each inhibitor. Each point represents the mean ± S.E.M. of 3 or 4 experiments.

Effect of Hybrid Depletion of MCT1 on Benzoic Acid Uptake by RBEC1

To evaluate the contribution of MCT1 to benzoic acid transport at the BBB, hybrid depletion of MCT1 in RBEC1 was performed using an antisense oligonucleotide against MCT1 (Fig. 5). When RBEC1 was transfected with the antisense oligonucleotide, [^{14}C]benzoic acid uptake was reduced to about 30% of the control, which is comparable with that observed in the presence of 10 mM benzoic acid. On the other hand, no change was observed in [^{14}C]benzoic acid uptake activity by RBEC1 transfected with the sense oligonucleotide against rat MCT1 (non-effective negative control).

Brain Uptake of [^{14}C]Benzoic Acid *In Vivo*

The brain uptake of [^{14}C]benzoic acid in rats was evaluated by means of an *in vivo* study using the BUI method (Fig. 6). Five seconds after intra-carotid injection, [^{14}C]benzoic acid (0.05 mM) uptake by the brain was about 74% of that of a reference compound, [^{3}H]water. In the concentration dependence study, the brain uptake of benzoic acid was saturable with *Km* of 1.27 ± 0.60 mM, *Vmax* of 371 ± 133 nmol/min/g and *kd* = 0.26 ± 0.02 ml/min/g. The *Km* value of benzoic acid uptake obtained by BUI was in good agreement with the results obtained in rat primary cultured BCECs and immortalized RBEC1.

DISCUSSION

Monocarboxylic acids such as lactic acid and pyruvic acid are presumed to be transported across the cell membranes of various tissues via members of the MCT transporter family (6,7). We have already demonstrated that some exogenous organic weak acids might be transported in a similar manner to native organic weak acids via MCT1 at the BBB, as well as in intestinal epithelial cells (8,16,29). As to monocarboxylic

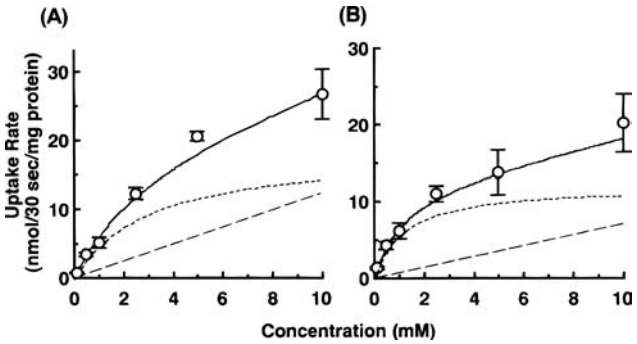

Fig. 3. Concentration dependence of benzoic acid uptake by cultured monolayers of rat primary BCECs (A) and immortalized BCECs (RBEC1) (B). The uptake of benzoic acid was determined at pH 6.0 and 37°C for 30 sec. Each point represents the mean ± S.E.M. of 3 or 4 experiments. The solid line represents total uptake rate. The dotted line represents the uptake rate for the saturable component calculated from the kinetic parameters obtained as mentioned under results. The dashed line represents nonsaturable uptake.

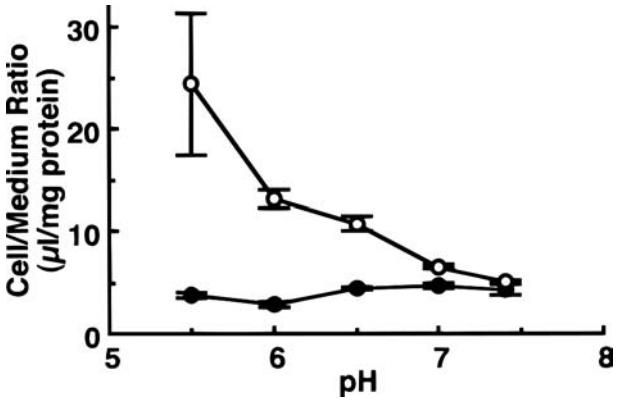

Fig. 4. pH Dependence of [^{14}C]benzoic acid uptake by cultured monolayers of rat immortalized BCECs (RBEC1). The uptake of [^{14}C]benzoic acid (100 μM) was determined at pH 5.5, 6.0, 6.5, 7.0 and 7.4 at 37°C for 30 sec in the absence (○) and presence (●) of unlabeled benzoic acid (10 mM). Each point represents the mean ± S.E.M. of 3 or 4 experiments.

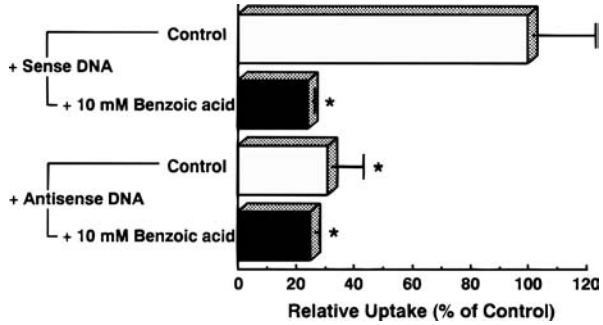

Fig. 5. Hybrid depletion of [^{14}C]benzoic acid uptake by cultured monolayers of rat immortalized BCECs (RBEC1) transfected with rat MCT1 antisense oligonucleotide. The uptake of [^{14}C]benzoic acid (42 μM) was determined at pH 6.0 and 37°C for 30 sec. Each point represents the mean ± S.E.M. of 3 or 4 experiments. *Significantly different from the control value ($p < 0.05$).

acid transport at the BBB, lactic and pyruvic acids have been shown to be transported by specialized mechanisms based on *in vivo* BBB transport studies (1,12,13). We also found that some organic weak acids such as salicylic and acetic acids may be transported via a transporter specific to monocarboxylic acids at the BBB by means of *in vivo* and *in vitro* methods (14,15), although the molecular identity of the transporters remains to be clarified. However, it is not necessarily appropriate to compare the underlying mechanism of the previously examined *in vivo* BBB transport with the *in vitro* experimental observations, because many of the *in vitro* BBB experiments were performed using bovine or hog cells, while the *in vivo* data were mainly from rats. Accordingly, in order to evaluate the functional significance of the monocarboxylic acid transporter MCT1 for the transport of exogenous organic weak acids, such as benzoic acid, at the BBB, in the present study we utilized rats and rat cell systems, because of the ease of handling *in vivo* and the substantial amount of information already available on rat membrane transporters at the molecular level.

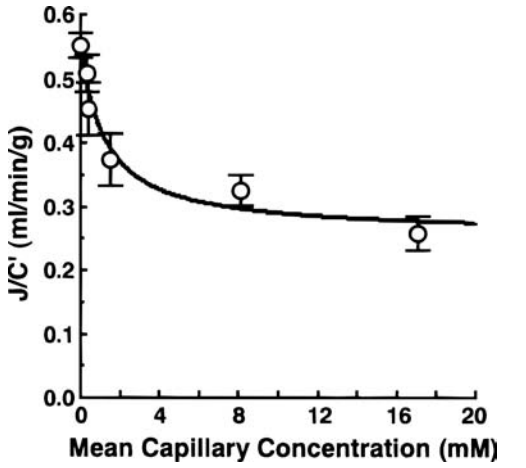

Fig. 6. Concentration dependence of brain uptake of [^{14}C]benzoic acid evaluated by the BUI method. Unidirectional clearance V/C′ is plotted versus the calculated mean capillary concentration of benzoic acid (C′). The line represents a computer-fitted result according to equation [5] as described in Materials and Methods. Each point represents the mean ± S.E.M. of 4–6 experiments.

Although a primary culture of rat BCECs has already been established (19), it has several practical disadvantages, such as a requirement for a large number of animals because of the poor yield, time-consuming preparation and poor reproducibility due to the low yield. Accordingly, in the present study, we established an immortalized rat cell line, RBEC1, which retains key features of BCECs, including moderate growth rate, spindle-shaped morphology, formation of a monolayer on collagen-coated dishes, and the presence of typical endothelial cell markers such as transferrin receptor antigen and γ-glutamyltranspeptidase activity. Furthermore, von Willebrand factor antigen and acetyl-LDL receptor activity were detected (date not shown). These observations indicated that the established cell line, RBEC1 is suitable for transport measurements as an *in vitro* rat BBB model.

We have already detected on the expression of MCT1 in rat brain capillaries by an RT-PCR method (8). Furthermore, a recent study demonstrated the expression of MCT1 in both luminal and abluminal plasma membranes of brain capillaries (9). So, in the present study we first of all confirmed the presence of rat MCT1 mRNA in RBEC1. As shown in Fig. 2, rat MCT1-specific primers produced a band of the expected size in RT-PCR of total RNA derived from RBEC1. Accordingly, RBEC1 expresses the MCT1 gene and is likely to be suitable for the functional analysis of monocarboxylic acid transport. Secondly, the uptake properties of L-[^{14}C]lactic acid were examined to confirm the functional expression of MCT1 in RBEC1. RBEC1 exhibited saturable uptake of L-lactic acid with a *Km* of 2.72 mM. This *Km* value is comparable with the value that we obtained in MDA-MB231 cells transfected with rat MCT1 (2.28 mM) (17) and with that of 1.8 mM determined by *in vivo* using the rat BUI method (1, 12). Furthermore, L-[^{14}C]lactic acid uptake by RBEC1 was temperature-dependent and was reduced by several monocarboxylic acids such as benzoic acid and mandelic acid, but not by a dicarboxylic acid, succinic acid (Table I). The inhibitory effects of lactic acid and mandelic acid were stereospecific, with L-lactic acid or S-mandelic acid showing higher affinity. These stereospecific and monocarboxylic acid-specific inhibitory effects are consistent with the known properties of MCT1-mediated transport of lactic acid (8,16,17). Therefore, it is concluded that RBEC1 can be used in the functional evaluation of monocarboxylic acid transport at the BBB.

We have already demonstrated that an exogenous monocarboxylic acid, benzoic acid, is a substrate of MCT1 (17), though no information on its BBB transport is available. Therefore, as a first step to evaluate the importance of MCT1 for the BBB transport of acidic drugs, we used benzoic acid as a model compound of anionic drugs containing a single carboxyl group. [^{14}C]Benzoic acid was efficiently taken up by rat brain *in vivo*, when evaluated by the BUI method at a tracer concentration (50 μM). The BUI value was similar to those of natural compounds such as D-glucose and amino acids that are well taken up by the brain (BUI values of phenylalanine and D-glucose were 0.55 and 0.33 times that of [^{3}H]water, respectively).

The uptake of benzoic acid by RBEC1 is saturable with a *Km* of 1.15 mM, which is comparable with that observed in primary cultures of rat BCEC, 2.89 mM. This *Km* value is also in good agreement with that of MDA-MB231 cells expressing rat MCT1 (3.05 mM) (17). In addition, *in vivo* evaluation of

benzoic acid transport at the BBB by the BUI method resulted in a *Km* of 1.27 mM, which is again comparable with those obtained *in vitro*, as described above. Furthermore, all of the monocarboxylic acids tested exhibited a significant inhibitory effect on [^{14}C]benzoic acid uptake by RBEC1 in a stereospecific manner, whereas dicarboxylic acid was not inhibitory. All of these observations imply that MCT1 plays an important role in the transport of benzoic acid at the BBB.

Next, we performed a hybrid depletion study to eliminate expression of MCT1 in RBEC1 by introducing an antisense oligonucleotide against MCT1 into the cells during their cultivation (Fig. 5). Uptake of [^{14}C]benzoic acid by RBEC1 transfected with an antisense oligonucleotide against rat MCT1 was markedly reduced and the inhibitory effect of unlabeled benzoic acid disappeared, whereas unlabeled benzoic acid-sensitive transport activity was maintained in the cells transfected with a sense oligonucleotide, which does not influence the expression of MCT1. Furthermore, uptake by the cells with the antisense oligonucleotide was comparable with that in the presence of 10 mM unlabeled benzoic acid. This observation may be ascribed to the blockade of MCT1 expression in RBEC1 cells by the antisense oligonucleotide against MCT1. Accordingly, no transporter other than MCT1 seems to be substantially involved in the uptake of benzoic acid in RBEC1 cells, though a contribution of minor transporters and/or a transporter with similar nucleotide sequences to MCT1, that would also hybridize with the antisense oligonucleotide, cannot be excluded.

As shown in Fig. 4 for [^{14}C]benzoic acid uptake and as reported previously, MCT1 is a proton-monocarboxylic acid cotransporter and is energized at acidic pH (7,8). Accordingly, a question arises as to how the MCT1-mediated transport of monocarboxylic acids from the blood circulation to endothelial cells is energized, because the physiological bulk pH of the blood is about 7.4 and is not favorable for MCT1 activity. MCT1-mediated transport at the BBB does seem to occur under physiological conditions, since a BUI study by Oldendorf *et al.* (12,13) showed that lactic acid was transported by a carrier-mediated transport mechanism at pH 7.55 in a saturable and stereoselective manner, as observed in the MCT1-mediated transport in the present study. There are at least two possible explanations. Firstly, MCT1 is still active at neutral pH, although its activity is lower than at acidic pH. Secondly, the pH might be more acidic in the close vicinity of the endothelial cells, since it has been reported that the Na^+/H^+ exchanger NHE-1 is present at endothelial cell membranes as detected by RT-PCR, and NHE-1 may supply protons to the MCT1 on the plasma membrane (30) in a similar manner to that reported for intestinal epithelial cells, which generate an acidic microclimate pH on the intestinal luminal side. Further study of the effect of pH on MCT1 activity at the blood-brain barrier is needed.

Unfortunately, since RBEC1 is unlikely to form tight junctions, the polarity of the monolayer have not been clarified yet, it is difficult to draw conclusions as to the directionality of the transport. MCT1 has been shown to exist on the abluminal membrane of the BBB as well as on the luminal blood-side membrane (9). Furthermore, we found that MCT1-mediated transport is bidirectional in MCT1 gene-transfected cells (17). Accordingly, MCT1 seems to mediate exchange of monocarboxylic acids between the bloodstream, intracellular space of BCEC and brain parenchymal extracellular space, depending on the relative concentrations of substrates, the pH and probably other factors.

In conclusion, in the present study we established an immortalized rat BCEC cell line, RBEC1, and showed that it is useful for *in vitro* BBB transport studies. We examined the transport of monocarboxylic acids at the BBB in rats by using the established RBEC1 and primary culture of rat BCECs as *in vitro* methods and BUI studies *in vivo*. The observed transport features were compared with our previous observations in cells transfected with rat MCT1. The good correlation of transport properties of lactic acid and benzoic acid among the various experimental methods used and the consistency of the results with previous findings suggest that MCT1 plays an important role in monocarboxylic acid transport at the BBB.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, by a grant from Tokyo Biochemical Research Foundation, by a grant from Japan Research Foundation for Clinical Pharmacology, by a grant from Takeda Science Foundation, and by the Japan Health Science Foundation, the Drug Innovation Project.

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