Delivery of Peptide Drugs to the Brain by Adenovirus-Mediated Heterologous Expression of Human Oligopeptide Transporter at the Blood-Brain Barrier

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ABSTRACT

The feasibility of using adenovirus-mediated human oligopeptide transporter (hPEPT1) gene transfer to achieve peptide drug delivery to the brain across the blood-brain barrier was tested by examining the accumulation of model peptides in a rat brain endothelial cell line (RBEC1) and rat brain after transduction with a recombinant adenovirus encoding hPEPT1-enhanced yellow fluorescent protein fusion gene (AdhPEPT1-EYFP). In vitro uptake of [3H]GlySar was determined in RBEC1 transduced with AdhPEPT1-EYFP. At pH 6.0, the uptake of [3H]GlySar by RBEC1 transduced with AdhPEPT1-EYFP was increased 4-fold compared with that of nontransduced cells. At pH 7.4, uptake of [3H]GlySar in AdhPEPT1-EYFP transduced RBEC1 was 1.5 times higher than that of nontransduced cells. Unlabeled glycylysarcosine (10 mM) reduced the uptake of [3H]GlySar to a level comparable with that of nontransduced cells. At 30 min after intravenous administration of cesfamoxil to rats transduced with AdhPEPT1-EYFP at 3.2 × 10⁹ plaque-forming units/rat by an in situ brain perfusion method, the brain-to-plasma concentration ratio (Kp) of cesfamoxil was increased about 2 times compared with that of nontransduced or AdGFP (control vector)-transduced rats, although this was not statistically significant. In contrast, Kp of [14C]inulin, a marker for extracellular fluid space, remained unchanged after adenoviral transduction. In conclusion, our results suggest that adenovirus-mediated heterologous expression of hPEPT1 in vivo could be a useful approach to deliver oligopeptides to the brain.

Peptides have multiple biological actions in the brain and are potentially valuable as neuropharmaceuticals in the treatment of various disorders of the central nervous systems (CNS) (Zlokovic, 1995). Possible roles of peptides in the CNS include 1) the involvement in neurotransmission and neuro-modulation, 2) the regulation of the neuroendocrine axis, 3) the regulation of cerebral blood flow, 4) the regulation of cerebral spinal fluid secretion, 5) the mediation of the integrity of the blood-brain barrier (BBB), 6) the modulation of the BBB permeability to nutrients, 7) the regulation of water and electrolyte contents of the brain, and 8) the regulation of the expression of specific proteins at the BBB. There are several disorders of the brain in which peptides are known to be implicated in the pathogenesis, such as Alzheimer’s disease, depression, stroke, and so on. Thus, peptide drugs may be useful to treat or to diagnose brain diseases. Delivery of peptide drugs to the brain, however, is an essential prerequisite for therapeutic effectiveness since distribution of peptides and proteins to the brain is generally very low because of the BBB that prevents many molecules from crossing into the brain. The anatomical basis of the BBB arises from special cellular features of brain capillary endothelial cells, which include tight junctions, and minimal pinocytosis and fenestration (Pardridge, 2002). The BBB is well known to have several transport systems that regulate the concentration and entry of solutes into the CNS (Tsuji and Tamai, 1999). They include 1) carrier-mediated transport, 2) receptor-mediated transcytosis, and 3) adsorptive-mediated transcytosis. Thus, various transporters and receptors are expressed at the BBB. It is difficult for peptides to penetrate the BBB, however, because

ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; hPEPT1, human H+ peptide cotransporter; RBEC, rat brain endothelial cell; CMV, cytomegalovirus; PFU, plaque-forming unit; MOI, multiplicity of infection; MES, 2-(N-morpholino)ethanesulfonic acid; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; EYFP, enhanced yellow fluorescent protein; HPLC, high-performance liquid chromatography; Kp, tissue-to-plasma concentration ratio; GFP, green fluorescent protein.
they are hydrophilic, biologically unstable, and usually large in size (Banks and Kastin, 1994).

The oligopeptide transporter PEPT1 accepts not only dipeptides and tripeptides as substrates but also peptide-mimetic drugs such as β-lactam antibiotics (Fei et al., 1994; Liang et al., 1995; Miyamoto et al., 1996; Sai et al., 1996; Tamai et al., 1997), angiotensin-converting enzyme inhibitors (Hu and Amidon, 1988), the antiviral drug valacyclovir (Balimane et al., 1998), and the anticancer drug bestatin (Saito and Inui, 1993). The physiological role of oligopeptide transporters lies in the (re)absorption of peptides from the intestinal and renal tubular lumen. Nevertheless, no transporter or transport activity for di- or tripeptides has been found at the BBB.

There have been several experimental trials aimed at using endogenously expressed oligopeptide transport activity for improving oral bioavailability (Tamai et al., 1998) or, for tumor targeting, using cultured cells such as human fibrosarcoma cell line HT-1080 (Nakanishi et al., 1997) and human pancreatic cell lines AsPc-1 and Capan-2 (Gonzalez et al., 1998) that express oligopeptide transport activity. We previously examined the feasibility of tumor-selective delivery of dipeptides and peptide-mimetic drugs by using the oligopeptide transport activity (Nakanishi et al., 2000). To our knowledge, however, there has been no experimental trial on drug delivery to the brain by using the activity of oligopeptide transporter in the BBB since it is not expressed at the BBB.

It was, therefore, the purpose of the present study to examine the feasibility of delivering peptide drugs to the brain by heterologous expression of human oligopeptide transporter at the brain capillary endothelial cells, which make up the BBB. In our previous study, we constructed a recombinant adenovirus containing human PEPT1 and enhanced yellow fluorescent protein (AdhPEPT1-EYFP) fusion gene (Toyobuku et al., 2002). In that study, heterologous expression of hPEPT1-EYFP in mouse liver greatly enhanced delivery of peptide-mimetics to the liver. In the present study, oligopeptide transport activity was assessed in a brain endothelial cell line, RBEC1, transduced with or without AdhPEPT1-EYFP in vitro. In addition, AdhPEPT1-EYFP was transduced into the brain of rats, and the distribution of cefadroxil, a substrate of the oligopeptide transporter hPEPT1, into the brain was evaluated in vivo.

**Materials and Methods**

**Materials.** Dulbecco’s modified Eagle’s medium, fetal calf serum, and nonessential amino acids were obtained from Invitrogen (Carlsbad, CA). α-Glucose was purchased from Wako Pure Chemical Industries (Osaka, Japan). Rat tail collagen (type I) was obtained from Collaborative Research, Inc. (Grand Island, NY). [3H]Glycylsarcosine (GlySar) (629 GBq/mmol) was purchased from Moravek Biochemical, Inc. (Mercury Lane, Brea, CA). [14C]Inulin (161 MBq/g) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Unlabeled glycylsarcosine and cefadroxil were purchased from Sigma-Aldrich (St. Louis, MO). Poly-l-lysine hydrochloride polymer, with an average molecular mass of 37 kDa (Sigma-Aldrich), was used as a cationic molecule. The protein assay kit was purchased from BioRad (Hercules, CA). All other chemicals were commercial products of reagent grade. The pBluescript II SK (-) vector containing human PEPT1 2.2-kilobase cDNA was a gift from Prof. F. H. Leibach (Medical College of Georgia, Augusta, GA).

**Construction of Recombinant Adenovirus Encoding hPEPT1-EYFP Fusion Gene.** The hPEPT1-EYFP fusion gene was constructed to express of hPEPT1 in in vitro-cultured cells or in vivo, as described previously (Toyobuku et al., 2002). The constructs were restriction enzyme-mapped and sequenced to confirm the correct insert orientation. The vector containing the hPEPT1-EYFP fusion gene was used to generate the replication-deficient recombinant adenovirus serotype 5 encoding AdhPEPT1-EYFP driven by a cytomegalovirus (CMV) promoter (Fig. 1). To generate pAdhPEPT1-EYFP, the 2.2-kilobase hPEPT1-EYFP insert digested with KpnI and transfected into HEK293 cells. For amplification, the recombinant adenovirus was propagated in HEK293 cells and purified by CsCl banding. AdhPEPT1-EYFP was stored at the concentration of 1.0 × 10^11 plaque-forming units (PFU)/ml.

**Cell Culture.** Rat brain capillary endothelial cells, RBEC1, were established by transfection of recombinant plasmids containing origin-defective simian virus 40 gene, SVori-8-16, into primary cultured rat brain capillary endothelial cells, as described previously (Kido et al., 2000). RBEC1 (1.0 × 10^5 cells/cm^2) were grown routinely in collagen type I-coated tissue flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) at 37°C under a 5% CO2/95% air atmosphere. The culture medium consisted of Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 20 mM NaHCO3, 150 µg/ml endothelial cell growth supplement, 5% fetal bovine serum, and 5% donor horse serum. The cells were seeded into four-well plates (1.0 × 10^5 cells/well) 3 days before adenovirus transduction and incubated at 37°C and 5% CO2.

**In Vitro Gene Transfer.** RBEC1 were plated onto rat tail collagen type I-coated four-well plates (Nunc Nueller to determine, Naperville, IL) for 3 days before transfection. Transduction of adenovirus was performed after the cells had reached 80 to 90% confluence, with AdhPEPT1-EYFP at the multiplicity of infection (MOI) of 100 in the presence of different ratios of poly-l-lysine. The uptake experiment was performed 1 or 3 days after transfection. Phase contrast and fluorescence images of the cells were obtained using a Zeiss Axiovert S100 microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with an appropriate filter.

**Uptake Experiment.** For the uptake study, RBEC1 (1.0 × 10^5 cells/well) were cultured at 37°C for 2 days on rat tail collagen type I-coated four-well plates after adenovirus transduction. When the cells reached confluence, they were washed three times with 1 ml of incubation solution (0.952 mM CaCl2, 5.36 mM KCl, 0.441 mM CaHPO4, 0.812 mM MgSO4, 136.7 mM NaCl, 0.385 mM Na2HPO4, 25 mM D-glucose, and 10 mM MES at pH 6.0 or 10 mM HEPES at pH 7.4) and preincubated at 37°C for 5 min. After the preincubation, 476 nM [3H]GlySar solution (0.25 ml) was added to initiate uptake. The cells were incubated at 37°C (physiological temperature) for the desired time, and then washed three times with 1 ml of ice-cold incubation solution to terminate uptake. To quantify [3H]GlySar

**Fig. 1.** Schematic representation of the pShuttle adenovirus vector encoding hPEPT1-EYFP fusion gene. The hPEPT1-EYFP cDNA was cloned into the KpnI/NcoI restriction site of the pShuttle-CMV vector.
in the cells, the washed cells were solubilized by the addition of 5 N NaOH (0.25 ml), followed by shaking for 2 h. The resultant lysates were neutralized with 5 N HCl and mixed with 4 ml of liquid scintillation cocktail, Cleasol (Nakalai Tesque, Kyoto, Japan). Radioactivity was determined using a liquid scintillation counter (LSC-1000; Aloka Co. Ltd., Tokyo, Japan). Cellular protein content was measured by the method reported previously (Bradford, 1976) using bovine serum albumin as a standard. Net uptake was expressed as the cell-to-medium ratio (micromoles per milligram of protein) obtained by dividing the uptake amount by the concentration of substrate in the incubation medium.

**In Vivo Gene Transfer to Anesthetized Rat.** All the animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals in the Takara-machi Campus of Kanazawa University. Male Wistar rats (6–8 weeks old; Japan SLC, Inc., Hamamtsu, Japan) used in this study had free access to food and water before sacrifice. Rats were anesthetized with an intramuscular administration of ketamine/xylazine (25/2.3 mg/kg). In vivo gene transfer to rat brain was carried out by the in situ brain perfusion technique using methods reported previously to increase the exposure of the BBB to adenovirus (Takasato et al., 1984). After exposure of the right carotid artery, the occipital and superior thyroid arteries were ligated and cut, and the right pterygopalatine artery was ligated. The right external carotid artery was catheterized for perfusion to the internal carotid artery with polyethylene tubing (SP-10; Natsume Seisakusho Co., Tokyo, Japan) filled with sodium heparin (100 IU/ml). AdhPEPT1-EYFP and AdGFP with different ratios of poly-L-lysine in storage buffer (50 mM NaCl, 0.05% bovine serum albumin, 25% glycerol, and 5 mM Tris at pH 8.0) were perfused by the infusion pump (model 22; Harvard Apparatus, South Natick, MA) at 0.06 ml/min for 24 s, with simultaneous ligation of perfused by the infusion pump (model 22; Harvard Apparatus, South Natick, MA) at 0.06 ml/min for 24 s, with simultaneous ligation of the right common carotid artery to prevent mixing with the systemic circulation. The infusion pump was followed by 5-min washes in PBS. Secondary antibody Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) diluted with 3% blocking agent in PBS at 1:200 was added to the primary C219 anti-mdr1 antibody-treated sample. Secondary antibody Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, Inc.) diluted with 3% blocking agent in PBS at 1:100 was added to the primary anti-EGFP antibody-treated sample. Incubation was then continued for 30 min. The slide glasses were washed as described above. They were mounted in VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA) and observed under a fluorescence microscope.

**Cefadroxil Disposition to the Brain in Anesthetized Rats Transduced with Adenovirus.** Rats were anesthetized with an intramuscular injection of ketamine/xylazine (25/2.3 mg/kg) 3 days after virus infection. An aliquot of saline solution containing test compound (cefadroxil; 40 mg/ml/kg) was injected through the external jugular vein. At 30 min after administration, blood was collected by decapitation. The right cerebral hemispheres were quickly isolated, weighed, and rinsed with ice-cold PBS. The tissues were homogenized in 10 mM MES buffer (pH 6.0). Then, 750 μl of acetonitrile was added to 250 μl of homogenized sample to extract the cefadroxil, and the sample was centrifuged at 12,000 rpm. The supernatant was evaporated and the residue was used as HPLC samples. Simultaneously, plasma was obtained by centrifugation of collected blood at 12,000 rpm. The concentration of cefadroxil in the samples was measured by HPLC. The HPLC system (Shimadzu Co., Kyoto, Japan) was equipped with a constant flow pump, LC6A, a UV detector, SPD-10A, a column oven, CTO-2A, and an integrator, Chromatopac CR6A. The analytical column was an Xterra MS C18 (Waters, Milford, MA). The mobile phase, a mixture of acetonitrile and water (3.75:96.25, v/v) containing 10 mM phosphoric acid adjusted to pH 3.0, was used at a flow rate of 0.8 ml/min. The effluent was detected at 240 nm.

All data are expressed as means ± S.E.M., and the number of experiments is shown with each result. The statistical analysis was performed by use of Student’s t test. The criterion of significance was taken to be P < 0.05.

**Results**

**Uptake of [3H]GlySar by RBEC1 Transduced with AdhPEPT1-EYFP.** To examine whether dipeptide transport activity is induced in the blood-brain barrier by AdhPEPT1-EYFP transduction, we evaluated [3H]GlySar uptake in transduced RBEC1 at pH 6.0. As can be seen in Fig. 2A, the uptake of dipeptides increased time dependently in AdhPEPT1-EYFP-transduced RBEC1, whereas that of nontransduced RBEC1 did not. RBEC1 transduced with AdhPEPT1-EYFP showed a 10 times higher uptake than that of nontransduced control cells at 5 min. On the other hand, when AdGFP was transduced into RBEC1 as a control vector at an MOI of 300, the uptake of [3H]GlySar was similar to that of nontransduced cells (Fig. 2B).

Unlabeled oligopeptide was included in the uptake buffer to test whether [3H]GlySar uptake was inhibitable or not (Fig. 2B). In the presence of unlabeled GlySar (10 mM) at a concentration above the apparent Kᵦ₀ (2.8 mM) of GlySar for PEPT1, uptake of [3H]GlySar in AdhPEPT1-EYFP-transduced RBEC1 was inhibited by over 80%, resulting in an uptake comparable with that of nontransduced RBEC1.

To confirm the pH dependent nature of oligopeptide transport activity in adenovirus-transduced RBEC1, uptake of [3H]GlySar was compared between pH 6.0 and 7.4 (Fig. 2B).
In AdhPEPT1-EYFP-transduced cells, uptake of $[^{3}H]$GlySar was 4 and 3 times greater than that of nontransduced cells at pH 6.0 and 7.4, respectively. The uptake of $[^{3}H]$GlySar at pH 6.0 was 1.3 times higher than that at pH 7.4. These results are consistent with the reported activity of $H^+$/peptide co-transporter (Fei et al., 1994; Liang et al., 1995).

Expression of hPEPT1-EYFP in RBEC1 after AdhPEPT1-EYFP Transduction. To investigate whether hPEPT1-EYFP transcript was expressed in RBEC1 after AdhPEPT1-EYFP transduction, RT-PCR was conducted using specific primers based on the nucleotide sequence of hPEPT1-EYFP (Fig. 3A). The PCR product corresponding to hPEPT1-EYFP was detected in AdhPEPT1-EYFP-transduced RBEC1. The expression of hPEPT1-EYFP fusion protein was examined by fluorescence microscopy in RBEC1. Those cells were transduced with AdhPEPT1-EYFP at an MOI of 300. As shown in Fig. 3B, there was no detectable expression of hPEPT1-EYFP, and fluorescence of hPEPT1-EYFP was below the detection level, which corresponds to the background level, despite the presence of mRNA signals.

Effect of Various Transduction Aids on the Expression of hPEPT1-EYFP in RBEC1. To improve the expression level of hPEPT1-EYFP fusion protein in RBEC1, we examined the effect of cationic transduction aids, cyclodextrin molecules, and polymer on the expression of hPEPT1-EYFP protein 24 h after adenovirus transduction by fluorescence microscopy (Fig. 4). Expression of hPEPT1-EYFP protein was detected at a higher level after AdhPEPT1-EYFP transduction in the presence of poly-L-lysine (Fig. 4B) or cyclodextrins (Fig. 4, C–G) than after transduction with AdhPEPT1-EYFP alone (Fig. 4A). β-Cyclodextrin most significantly enhanced adenoviral-mediated gene transfer to RBEC1 among the cyclodextrins (Fig. 4D). Nevertheless, cyclodextrin mildly disrupted the cell membrane and altered the shape of RBEC1 observed in the phase-contrast image (data not shown). On the other hand, addition of poly-L-lysine caused no apparent change of cell shape. The use of Poloxamer 407, a viscous biocompatible polyol, as a transduction aid did not affect the expression of hPEPT1-EYFP compared with adenovirus alone (Fig. 4H). So, poly-L-lysine was suggested to improve the efficiency of transduction.

Effect of Addition Ratio of Poly-L-Lysine during Transduction on the Expression of hPEPT1-EYFP in RBEC1. To find the optimal conditions regarding poly-L-lysine, we prepared mixtures of adenovirus with poly-L-lysine—lysine, we prepared mixtures of adenovirus with poly-L-lysine—lysine, we prepared mixtures of adenovirus with poly-L-lysine—lysine, we prepared mixtures of adenovirus with poly-L-lysine—lysine, we prepared mixtures of adenovirus with poly-L-lysine—lysine, we prepared mixtures of adenovirus with poly-L-lysine—lysine, we prepared mixtures of adenovirus with poly-L-lysine—lysine. The expression level of hPEPT1-EYFP increased with an increasing ratio of poly-L-lysine to adenovirus during transduction (Fig. 5). The expression level of hPEPT1-EYFP increased with an increasing ratio of poly-L-lysine to adenovirus during transduction (Fig. 5). The expression level of hPEPT1-EYFP increased with an increasing ratio of poly-L-lysine to adenovirus during transduction (Fig. 5). The expression level of hPEPT1-EYFP increased with an increasing ratio of poly-L-lysine to adenovirus during transduction (Fig. 5). The expression level of hPEPT1-EYFP increased with an increasing ratio of poly-L-lysine to adenovirus during transduction (Fig. 5). The expression level of hPEPT1-EYFP increased with an increasing ratio of poly-L-lysine to adenovirus during transduction (Fig. 5). The expression level of hPEPT1-EYFP increased with an increasing ratio of poly-L-lysine to adenovirus during transduction (Fig. 5).
Expression of hPEPT1-EYFP in Rat Brain after AdhPEPT1-EYFP Transduction.

To investigate whether hPEPT1-EYFP transcript was expressed in vivo after administration of AdhPEPT1-EYFP to rat, RT-PCR analysis of hPEPT1-EYFP was conducted. After transduction at the virus dose of $3.2 \times 10^9$ PFU/rat, mRNA of hPEPT1-EYFP was detected in the rat brain (Fig. 6A). In addition, to evaluate whether hPEPT1-EYFP protein was induced in vivo after AdhPEPT1-EYFP transduction, expression in brain was examined by fluorescence microscopy of brain slices (Fig. 6B). At the virus dose of $3.2 \times 10^9$ PFU/rat, hPEPT1-EYFP fluorescence was observed (Fig. 6B, a), whereas no fluorescence signal was observed in the case of transduction without AdhPEPT1-EYFP (Fig. 6B, a).

The localization of hPEPT1-EYFP was examined by immunofluorescence studies of rat brain cryosections (Fig. 6B, b). The endothelial membrane marker P-glycoprotein was immunostained with monoclonal antibody C219 as the primary antibody. hPEPT1-EYFP fusion protein was detected with anti-EGFP antibody (Fig. 6B, c). Morphologically, the distribution of hPEPT1-EYFP fluorescence appeared to be at least partly colocalized with that of P-glycoprotein.

Drug Disposition to the Brain of AdhPEPT1-EYFP-Transduced Rat.

To evaluate whether the disposition of dipeptides was improved after AdhPEPT1-EYFP transduction of rats, the distribution of cefadroxil to the brain was measured at 30 min after administration, and Kp values, which were obtained by dividing the total concentration of cefadroxil in the brain by that in plasma, were evaluated. $[^{14}C]$Inulin was used to estimate the distribution in the extracellular fluid space because it hardly enters the cell (Tsuji et al., 1983). As shown in Fig. 7, in the brain of rats transduced with AdhPEPT1-EYFP at the dose of $3.2 \times 10^9$ PFU/rat, the Kp value of cefadroxil was increased about 2 times compared with that in nontransduced rats, although this was not statistically significant. There was no significant difference between the Kp values of $[^{14}C]$inulin in nontransduced and AdhPEPT1-EYFP-transduced rat brain (data not shown). When AdGFP was infected as a control vector, no significant increase in Kp of cefadroxil in the brain was observed, and the value was comparable to that of nontransduced rats.

Discussion

The entry of most polar molecules and proteins into the brain from circulating blood is restricted by the endothelial wall, that is, the BBB. Peptides do not readily penetrate the BBB because they are hydrophilic, biologically unstable, large molecules. Even small di- or tripeptides, such as β-lactam antibiotics, that are used for the treatment of central nervous system infections, seem to cross the blood-brain barrier in only negligible amounts, which do not differ from those of extracellular markers (Torok et al., 1998). PEPT1 or PEPT2 expressed in intestinal and renal epithelial cells transport small peptides such as di- and tripeptides. It has not been clarified, however, whether peptide transporters...
exist at the BBB. Therefore, in the current study, we attempted to induce expression of the oligopeptide transporter hPEPT1 in rat endothelial wall, by using a recombinant adenovirus, to examine the feasibility of this approach for drug delivery to the brain.

Craniotomy-based drug delivery, including either intraventricular drug infusion or local intracerebral implants, can be used to deliver a very small volume ($\leq 1 \text{ mm}^3$) from a local depot site (Partridge, 2002). It is desirable, however, to develop a system to transport target molecules across the BBB and supply them widely throughout the brain after intravenous administration. Therefore, we have attempted to express human oligopeptide transporter at the BBB to use its transport activity for the delivery of drugs to the brain. For delivery of drugs from the systemic circulation to the brain across the BBB under physiological conditions, we must take into consideration that the pH of the systemic circulation is 7.4. In our study, significant uptake of $[^3\text{H}]\text{GlySar}$ in AdhPEPT1-EYFP-transduced BBB model cells, RBEC1, was observed at pH 7.4, although the activity was lower than that at acidic pH (Fig. 2). Transport activity of hPEPT1 at neutral pH has also been reported by other investigators. Transport of glycylsarcosine in $X. \text{ laevis}$ oocytes injected with hPEPT1 cRNA was observed at pH 7.5 (Liang et al., 1995). Uptake of $[^3\text{H}]\text{GlySar}$ in Chinese hamster ovary cells stably expressing hPEPT1 was also observed at pH 7.5 (Covitz et al., 1996). Gonzalez et al. (1998) reported transport activity of $[^3\text{H}]\text{GlySar}$ in AsPc-1 and Capan-2 cells at pH 7.4, in accordance with the present findings. In our previous report, uptake of the dipeptide carnosine was observed at pH 7.4 in AdhPEPT1-EYFP-transduced WIFB9 cells used as a model of polarized liver cells (Toyobuku et al., 2002). Furthermore, Guo et al. (1999) reported that the optimum pH for valacyclovir uptake was pH 7.5. On the other hand, it has been reported that Na$^+/\text{H}^+$ antiporter (NHE1) is present at the endothelial cell membrane (Kalaria et al., 1998). Thus, the environment might be made more acidic in the close vicinity of the endothelial cells by the action of NHE1. In any event, hPEPT1-EYFP fusion protein was functional in medium of neutral pH in BBB model cells transduced with AdhPEPT1-EYFP.

It is generally difficult to transfer a foreign gene to the blood vessels from the systemic circulation in vivo. In our present study, the expression level of hPEPT1-EYFP fusion

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**Fig. 6.** A, RT-PCR analysis of mRNA level of hPEPT1-EYFP in right brain after adenovirus-transduction of rat. Expression of hPEPT1-EYFP mRNA in rat transduced with AdhPEPT1-EYFP at the dose of $3.2 \times 10^9 \text{ PFU/rat}$. Total RNA extracted from rat right brain after 3 days of transduction was subjected to RT-PCR using primer pairs specific for hPEPT1-EYFP. The RT-PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. RT (−) represents the control lacking reverse transcriptase enzyme. B, Immunohistochemistry of hPEPT1-EYFP fusion protein in rat brain slices after adenovirus transduction. A semithin cryosection (10 μm) of the rat brain transduced with AdhPEPT1-EYFP at the dose of $3.2 \times 10^9 \text{ PFU/rat}$ was subjected to indirect immunofluorescence microscopy. Nontransduced right brain of rat was used as a control (a). Anti-EGFP antibody was used to identify expression of hPEPT1-EYFP in brain capillary endothelial cells (b). C219 monoclonal antibody against P-glycoprotein was used to identify brain endothelial domain (c). Bar = 20 μm. bp, base pair.

**Fig. 7.** $K_p$ value of cefadroxil in rat brain without transduction (open column) or with transduction of AdhPEPT1-EYFP (closed column) and AdGFP (striped column) at the dose of $3.2 \times 10^9 \text{ PFU/rat}$. $K_p$ values were obtained 30 min after intravenous administration of cefadroxil in rat with or without transduction of adenovirus. Each value represents the mean $\pm$ S.E.M. of three to five rats.
protein in RBEC1 was very low, although mRNA of hPEPT1-EYFP was observed and was functional in vitro (Fig. 3). Nevertheless, the expression level of hPEPT1-EYFP in RBEC1 was improved when a cationic polymer, such as poly-L-lysine, was included during the transfection process (Fig. 4B). Toyoda et al. (1998) reported a similar result in vascular cells. We also demonstrated that the expression level of hPEPT1-EYFP in rat brain in vivo was improved by the presence of poly-L-lysine during transfection (Fig. 6). This may be due to enhancement of the electrostatic interaction of adenosine virus with the cell surface, favoring subsequent virus-mediated steps. Croyle et al. (1998) reported that the expression level of GFP in rat small intestine was improved when β-cyclodextrin was used during the transduction (Croyle et al., 1998). Cyclodextrins significantly reduced transepithelial electrical resistance, however. We found that the morphology of RBEC1 observed under a phase-contrast microscope was also observed in RBEC1, however, probably due to the removal of cholesterol from the cell membrane by β-cyclodextrin. Therefore, we selected poly-L-lysine to improve the expression level of hPEPT1-EYFP in the in vitro and in vivo experiments.

The localization of hPEPT1-EYFP is critical if the transporter is to deliver peptide drugs from the systemic circulation into the brain. Membrane transporters, such as GLUT1 or choline transporter at the BBB, take up drugs or endogenous compounds from the circulation in the brain. Our results showed that hPEPT1-EYFP was expressed in RBEC1 after adenovirus transduction (Fig. 4B). Yet, the localization of hPEPT1-EYFP was not clear in this study. Sun et al. (2001) detected hPEPT1-GFP at the apical and basolateral membranes of Caco-2 cells (Sun et al., 2001). It was reported that hPEPT1 was present in the plasma membrane and intracellular vesicular structures of AsPC-1 and Capan-2 cells (Gonzalez et al., 1998). hPEPT1 is localized in nuclei of vascular smooth muscle cells and lysosomes of the exocrine pancreas (Bockman et al., 1997). Thus, the distribution of hPEPT1 in various cells is diverse. In our present study, the Kp value of cefadroxil in AdhPEPT1-EYFP-transduced rat brain was increased after intravenous injections. Therefore, the hPEPT1-EYFP fusion protein is considered to be functional at least in the luminal membrane of the rat BBB. This is desirable for delivery of peptide drugs from the systemic circulation into the brain. Significant cytoplasmic staining was also observed in RBEC1; however, probably due to the overexpression of the transporter. Further investigation on colocalization of hPEPT1-EYFP with various subcellular markers is needed.

Recently, Berger and Hediger (1999) reported that PEPT2 mRNA is expressed in brain, especially in astrocytes, subependymal cells, ependymal cells, and epithelial cells of the choroid plexus. Furthermore, the peptide transporter PEPT2 was recently shown to be functional in rat choroid plexus, suggesting that it may play a role in neuropeptide homeostasis in the cerebrospinal fluid (Teuscher et al., 2001). Another oligopeptide transporter, PHT1, responsible for transport of histidine oligopeptides and histidine itself, has been cloned from brain (Yamashita et al., 1997). Recently, PTR4 was cloned from human brain (GenBank accession no. AY038999). It has not been clarified yet whether PEPT2, PHT1, or PTR4 exists at the BBB, however. In our study, [3H]GlySar uptake by BBB model cells in vitro was increased after AdhPEPT1-EYFP transduction. Furthermore, the Kp value of cefadroxil in AdhPEPT1-EYFP-transduced rat brain was slightly increased compared with that of nontransduced or AdGFP transduced brain, although without statistical significance. These results indicated that delivery of peptide drugs to the brain across the BBB is possible by heterologous expression of hPEPT1-EYFP after transduction with an adenovirus vector.

Current treatment methods for brain cancer are still inadequate. Malignant gliomas are the most common primary neoplasms of the central nervous system, and the prognosis for patients diagnosed with high-grade glioma (glioblastoma multiforme) remains bleak; survival is for less than 1 year (Saleh et al., 2000). Therefore, new therapeutic strategies need to be developed for gliomas. It was reported that metalloprotease inhibitors, including puromycin and bestatin, induce apoptosis in glioma cells (Schlapbach and Fontana, 1997). Bestatin is an anticancer agent that is transported by PEPT1. Although, hPEPT1 is not expressed in the brain tissues (Liang et al., 1995), heterologous expression of PEPT1 at the BBB may allow bestatin to enter the brain efficiently across the BBB. Furthermore, this system could also be applicable to a new melphalan produg designed for tumor-selective activation (Kerr et al., 1998). It is not clear, however, whether heterologous expression of PEPT1 by adenovirus is safe for treatment of disease. The drug delivery system reported here might be applicable to the treatment of the brain tumors if less toxic and more effective vectors are developed.

We think that the feasibility of this approach for human therapeutic intervention is dependent not only on the safety of transient expression of hPEPT1 by adenovirus but also on whether any alternative is available to the patient. Many pharmacologically effective peptide-mimetic drugs have been developed, but the BBB permeability of peptides is extremely low, even though expression of some peptide transporter genes at the BBB has been reported. Importantly, the substrate recognition spectrum of oligopeptide transporter hPEPT1 is extremely broad. As reported recently, PEPT1 accepts not only traditional peptide-mimetic agents, but also valacyclovir, 4-aminophenyl, δ-aminolevulinic acid, 6-amino-hexanoic acid, and so on. These observations suggest that a peptide bond is not essential for substrates of this transporter (Balimane et al., 1998). Accordingly, novel nonpeptide PEPT1 substrates with pharmacological activity in the brain could also be candidates for this approach.

BBB-specific exogenous gene expression will provide unique opportunities to deliver drugs from the systemic circulation to the brain by specific expression of a peptide transporter at the BBB and also to study the physiological function of the BBB. The expression of BBB markers, for example GLUT1, has been investigated (Board, 1996). The criterion for a BBB-specific gene is expression principally, although not necessarily exclusively, at the BBB (Partridge et al., 1990). Papoutsi et al. (2000) reported that tumor cells induced the expression of neurothelin/HT7 (but not of glucose transporter-1), a marker of blood-brain-barrier-forming endothelial cells. Regulation of such BBB-specific genes by promoters or enhancers is poorly understood. Promoters are an important determinant of the amount and rate of expression after gene transfer. In our present study, we used adenovirus...
vectors driven by CMV (cytomegalovirus) promoter. The CMV major immediate early promoter/enhancer drives constitutive expression, and response elements within the enhancer allow inducible expression through binding of active transcription factors. Thus, site-specific expression of an exogenous gene might be attained by replacing the CMV promoter with a blood-brain-barrier-specific promoter.

The onset and duration of transgene expression are important to evaluate the usefulness of a selected transporter in normal rats. In our previous experiment, the expression of AdGFP was 4 weeks in mouse liver (data not shown). In the present study, we focused on evaluating the expression and function of hPEPT1 in the rat brain 3 days after adenoviral transduction. Further investigation of the onset and duration of transgene expression is required to optimize the concentration of hPEPT1 substrates in the brain. Furthermore, better methods are required to control the transgene expression.

In conclusion, we have demonstrated the feasibility of a novel strategy for enhanced drug delivery to the brain by using adeno-virus-mediated heterologous expression of an oligopeptide transporter gene. This system is expected to be applicable for the studies of the function of human transporter genes in animal models. This system may also be applicable to chemically modified substrates that are recognized by PEPT1 because PEPT1 accepts a wide range of peptide and peptide-like substances as substrates.

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References

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