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Functional characterization of the brain-to-blood efflux clearance of human amyloid-β peptide (1–40) across the rat blood–brain barrier

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Abstract

The present study sought to characterize the brain-to-blood efflux transport of human amyloid-β peptide (hAβ)(1–40) across the blood–brain barrier (BBB) in rats. We determined the apparent brain-to-blood [^{125}I]hAβ(1–40) efflux clearance in rats and found it to be 11.0 μL/(min g brain). There were no significant gender differences in the apparent brain-to-blood [^{125}I]hAβ(1–40) efflux clearance. The brain-to-blood [^{125}I]hAβ(1–40) efflux transport was significantly inhibited by unlabeled hAβ(1–40) and hAβ(1–42) by 79.1% and 36.4%, respectively, but was not inhibited by hAβ(1–43) and hAβ(40–1), and was significantly facilitated by hAβ(17–40) by 16.0%, which is one of the major proteolytic fragments of hAβ(1–40) generated by the action of Aβ degradation enzymes, such as endothelin-converting enzyme. Pre-administration of human receptor-associated protein, a low-density lipoprotein receptor-related protein (LRP) antagonist, reduced the elimination of [^{125}I]hAβ(1–40) by 20.3%, while quinidine or verapamil, P-glycoprotein (P-gp) inhibitors, did not significantly affect the elimination. Western blot analysis suggested that LRP-1 is expressed in rat brain capillary endothelial cells. In conclusion, the partial contribution of LRP-1 and the minor contribution of P-gp suggest that the hAβ(1–40) elimination from rat brain is mediated by as yet unidentified molecules.

Keywords: Alzheimer's disease; Aβ degrading enzyme; Efflux transport; Gender difference; LRP-1; P-gp

1. Introduction

An abnormally elevated level of amyloid-β peptide (Aβ) in the brain is one of the prominent features of Alzheimer's disease (AD) (Hardy and Selkoe, 2002). Aβ is a 38–43 amino acid peptide derived from the proteolytic processing of amyloid precursor protein (APP), and Aβ(1–40) is the major component of amyloid plaques in brain. When [^{125}I] human Aβ(1–40) (hAβ(1–40)) was microinjected into the brain, it was eliminated from the brain to the blood via the blood–brain barrier (BBB) (Shibata et al., 2000; Shiiki et al., 2004), which consists of brain capillary endothelial cells (Terasaki and Ohtsuki, 2005). This elimination process could play an important role in preventing the accumulation of hAβ(1–40) in the brain. On the other hand, exogenous [^{125}I]hAβ(1–40) administered to the circulating blood or via the carotid artery was transferred into the brain (Martel et al., 1997; Wengenack et al., 2000; Deane et al., 2003), suggesting that peripheral hAβ(1–40) is also transported into the brain across the BBB. Therefore, both blood-to-brain and brain-to-blood transport determine the net flux of Aβ transport across the BBB and would play an important role in the accumulation of cerebral Aβ.

In vivo cerebral Aβ clearance has been reported to involve a specific receptor (low-density lipoprotein receptor-related protein 1 (LRP-1)) (Shibata et al., 2000) and proteases (neprilysin (NEP) (Iwata et al., 2001), insulin-degrading enzyme (IDE) (Farris et al., 2003) and endothelin-converting enzyme (ECE) (Eckman et al., 2003)). Since the involvement of each of these in cerebral hAβ(1–40) clearance has been examined in different in vivo systems, the contribution that each of these molecules makes to the clearance system is still unclear. Indeed, it has been reported that LRP-1 is involved in hAβ(1–40) efflux transport in the mouse brain using human receptor-associated protein (RAP) (Shibata et al., 2000), while

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our recent report failed to demonstrate a significant contribution of LRP-1 to hAβ(1–40) efflux transport in the rat brain using rat RAP (Shiiki et al., 2004).

P-glycoprotein (P-gp) is expressed on the luminal membrane of brain capillary endothelial cells (BCEC) and excretes substrates into the circulation. Recent studies have reported that hAβ(1–40) and hAβ(1–42) could be substrates of P-gp (Lam et al., 2001) and the expression levels of P-gp inversely correlate with Aβ deposition in the brain in elderly humans and P-gp knockout (*multidrug resistance 1a/b*$^{-/-}$) mice (Vogelgesang et al., 2002; Cirrito et al., 2005). However, P-gp gene deficiency reduced the expression level of LRP-1 in brain capillaries (Cirrito et al., 2005). Furthermore, since P-gp is expressed in the liver which is the major organ responsible for systemic hAβ(1–40) clearance (Ghiso et al., 2004), P-gp gene deficiency is likely to affect systemic hAβ(1–40) clearance. Therefore, it is still unknown whether P-gp directly contributes to brain-to-blood transport of hAβ(1–40) at the BBB in vivo. It is of crucial importance to characterize in vivo BBB Aβ efflux transport and, particularly, the contribution of LRP-1 and/or P-gp as part of the efflux system preventing the accumulation of Aβ in the brain.

The purpose of this study was to clarify the brain-to-blood efflux clearance of hAβ(1–40) by combining brain efflux index (BEI) and brain slice uptake studies. We also investigated the contribution of LRP-1 and P-gp to cerebral Aβ clearance in rats by means of a BEI study.

2. Materials and methods

2.1. Animals

Male and female Sprague–Dawley rats (7–8 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). All experiments were approved by the Animal Care Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University.

2.2. Reagents

Monoiodinated and non-oxidized [^{125}I]hAβ(1–40) (2200 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). [^{3}H]Dextran was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). [carboxy-^{14}C]inulin ([^{14}C]inulin, 1.92 mCi/g) was purchased from ICN Biochemicals (Costa Mesa, CA, USA). The unlabeled hAβ peptides and fragments were purchased from Bachem (Bubendorf, Switzerland) and Sigma (St. Louis, MO, USA). The human RAP was purchased from Oxford Biochemical Research (Oxford, MI, USA). Xylazine hydrochloride was purchased from Sigma. Ketaral 50 (ketamine hydrochloride) was obtained from Sankyo Co. (Tokyo, Japan). All other chemicals were analytical grade commercial products.

2.3. BEI study

The in vivo brain elimination experiments were performed using the intracerebral microinjection technique (Kakee et al., 1996). In brief, rats were anesthetized with an intramuscular injection of xylazine (1.22 mg/kg) and ketamine (125 mg/kg), and placed in a stereotaxic frame (SR-6; Narishige, Tokyo, Japan), which determines the coordinates of the rat brain which coincide with parietal cortex area 2 (Par2). A burr hole was made 5.5 mm lateral and 0.2 mm anterior to the bregma, and a fine injection needle was advanced to a depth of 4.5 mm. The applied solution (0.50 μL) containing [^{125}I]hAβ(1–40) (0.02 μCi) and [^{3}H]dextran (0.20 μCi) in an extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM HEPES, pH7.4) was administered into the Par2 region over a period of 30 s. The [^{125}I]hAβ(1–40) in the applied solution was in monomeric form as confirmed by gel filtration chromatography (data not shown). After microinjection, the microsyringe was left in place for 4 min to minimize any backflow. At designated times, the left and right cerebrum and cerebellum were excised and dissolved in 2.5 mL 2 M NaOH at 60 °C for 1 h. The [^{125}I] radioactivity of the samples was measured in a γ-counter (ART300, Aloka, Tokyo, Japan) for 3 min. The samples were then mixed with 14 mL Hionic-fluor (Packard Instruments, Meriden, CT, USA), and the [^{3}H] radioactivity was measured in a liquid scintillation counter (TRI-CARB2050CA, Packard Instruments) for 5 min. The BEI was defined by Eq. (1), and the percentage of substrate remaining in the ipsilateral cerebrum (100 − BEI) was determined using Eq. (2):

$$\mathrm{BEI}\,(\%) = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \quad (1)$$

$$100 - \mathrm{BEI}\,(\%) = \frac{\text{amount of test substrate in the brain}\,/\,\text{amount of reference in the brain}}{\text{concentration of test substrate injected}\,/\,\text{concentration of reference injected}} \times 100 \quad (2)$$

The apparent elimination rate constant ($K_{app,el}$) was determined from the slope given by fitting a semilogarithmic plot of (100 − BEI) versus time, using the nonlinear least-squares regression analysis program MULTI (Yamaoka et al., 1981). The apparent brain-to-blood efflux clearance across the BBB, $CL_{BBB,efflux}$, is obtained from

$$CL_{BBB,efflux} = K_{app,el} \times V_{brain} \quad (3)$$

where V_{brain} represents the distribution volume, determined by an in vitro brain slice uptake study as described below.

To characterize the efflux transport system at the BBB, the BEI value of [^{125}I]hAβ(1–40) at 60 min was determined by co-administration or pre-administration of several inhibitors. In the co-administration study, the concentration of co-administrated inhibitors in brain was diluted 30.3-fold compared with that in the injectate as reported previously (Kakee et al., 1996). When the inhibitor concentration at the microinjection site could not be maintained high enough by co-administration, a sufficient volume (50 μL) of the inhibitor solution was pre-injected at the microinjection site of [^{125}I]hAβ(1–40) to minimize the dilution effect (Kakee et al., 1996). The BEI value at 60 min when 50 μL of ECF buffer was pre-injected was not significantly different from that of the co-administered control ($p = 0.06$) (Table 1). Our previous study also demonstrated that the pre-administration of 50 μL solution did not affect the elimination rate of [^{125}I]hAβ(1–40) from the rat brain (Shiiki et al., 2004).

2.4. Determination of the distribution volume of [^{125}I]hAβ(1–40) in the brain

The distribution volume of [^{125}I]hAβ(1–40) in the brain was determined in a series of in vitro uptake experiments using brain slices, as described previously (Kakee et al., 1996). In brief, the brains were immediately removed after the rats were decapitated and the brains then were dissected in ice-cold oxygenated ECF buffer. Hypothalamic slices (300-μm thick) were cut using a brain microslicer (DTK-2000, Dosaka EM Co., Kyoto, Japan), and kept in an oxygenated ECF buffer equilibrated with 95% O_2–5% CO_2. After pre-incubation for 5 min at 37 °C, the brain slices were transferred to 5 mL oxygenated ECF buffer containing 0.02 μCi/mL [^{125}I]hAβ(1–40) or 0.01 μCi/mL [^{14}C]inulin at 37 °C. At appropriate times, brain slices and samples of the incubation medium were collected. The [^{125}I] and [^{14}C] radioactivities were measured using a γ-counter and a liquid scintillation counter, respectively. The apparent zero-time intercept of the uptake time profile of [^{14}C]inulin (i.e., 0.166 for males and 0.161 for females mL/g brain) was used to correct for the adherent water volume.

2.5. Western blot analysis

The crude membrane and whole cell lysate fractions of rat brain and the rat brain capillary-rich fraction were prepared using the procedure described in a

Table 1
Effects of hAβ peptides and fragments on [^{125}I]hAβ(1–40) elimination from rat brain

Peptides	Concentration in injectate (μM)	No. of studied	BEI (%)	% of control
(A) Co-administration				
Control		7	63.6 ± 1.7	100
hAβ(1–40)	20	4	13.5 ± 8.2**	21.2
hAβ(40–1)	20	4	62.6 ± 2.0	98.5
Control (2% DMSO)		7	62.2 ± 2.3	100
hAβ(1–42)[a]	20	9	61.0 ± 1.4	98.1
hAβ(1–16)[a]	20	4	70.7 ± 3.7	114
hAβ(1–28)[a]	20	4	64.8 ± 1.0	104
hAβ(17–40)[a]	20	4	72.2 ± 1.9*	116
hAβ(29–40)[a]	20	3	66.4 ± 1.8	107
(B) Pre-administration				
Control (0.1% DMSO)		6	57.7 ± 2.2	100
hAβ(1–40)[b]	1	4	12.0 ± 5.1**	20.9
hAβ(40–1)[b]	1	3	50.9 ± 3.1	88.3
hAβ(1–42)[b]	1	4	36.7 ± 5.4**	63.6
hAβ(1–43)[b]	1	5	49.2 ± 4.5	85.3

(A) [^{125}I]hAβ(1–40) (0.02 μCi) and [^{3}H]dextran (0.20 μCi) dissolved in 0.50 μL ECF buffer was injected into male rat brain in the presence or absence of 20 μM of each hAβ peptide/fragment.

[a] hAβ peptide solution containing 2% DMSO was administered. [^{125}I]hAβ(1–40) was used at a concentration of 18.2 nM (i.e. 0.6 nM as the cerebral concentration). (B) Fifty microliters of 1 μM hAβ peptide solution was pre-injected 5 min before administration of [^{125}I]hAβ(1–40) (0.02 μCi) and [^{3}H]dextran (0.20 μCi) into the same brain region.

[b] hAβ peptide solution containing 0.1% DMSO was administered. The value was determined 60 min after intracerebral administration. Each value represents the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from each control.

previous report (Hosoya et al., 2000). The protein samples were incubated at 95 °C for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20 μg per lane). Proteins were electroblotted onto a nitrocellulose membrane. The membrane was treated with a blocking buffer (4% skimmed milk in 25 mM Tris–HCl (pH8.0), 125 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with antibodies against the β-chain of LRP-1 (0.1 μg/mL; 3501, American Diagnostica Inc., Stamford, CT, USA) or P-gp (4 μg/mL; C219, Signet Laboratories, Dedam, MA, USA) as the primary antibody at 4 °C for overnight. The membrane was washed three times with blocking buffer and incubated with a horseradish peroxidase-conjugated second antibody. The bands were visualized with an enhanced chemiluminescence kit (SuperSignal; Pierce, Rockford, IL, USA).

2.6. Data analysis

$K_{app,el}$ and brain-to-blood hAβ(1–40) efflux clearance represent the mean ± S.D. Other data represent the mean ± S.E.M. An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between two group means. One-way analysis of variance followed by Dunnett's test was used to assess the statistical significance of differences among means of more than two groups.

3. Results

3.1. The apparent brain-to-blood efflux clearance of [^{125}I]hAβ(1–40) in rats

The in vivo brain-to-blood efflux transport of [^{125}I]hAβ(1–40) was determined using the BEI method with [^{3}H]dextran as a reference compound. [^{125}I]hAβ(1–40) was injected into rat brain at a concentration of 18.2 nM in the injectate (0.6 nM as the cerebral concentration), which was lower than the half

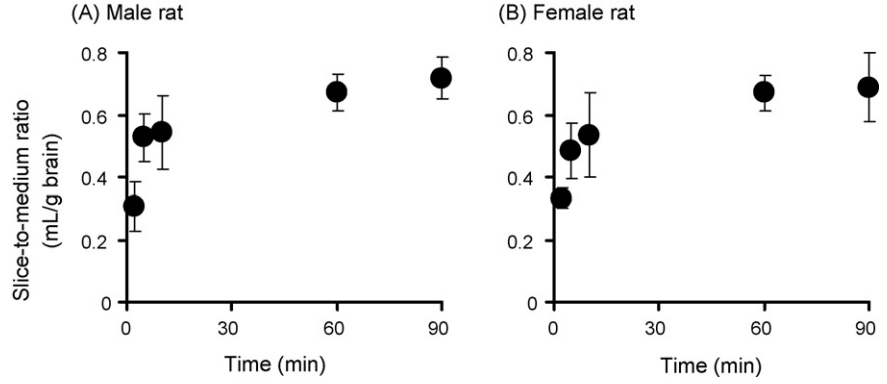

Fig. 1. Time courses of the apparent uptake of [^{125}I]hAβ(1–40) by rat brain slices. Male (A) and female (B) rat brain slices were incubated with 0.02 μCi/mL [^{125}I]hAβ(1–40) (1.8 pM) at 37 °C. At the indicated times, the radioactivity in the brain slices and incubation medium were measured, and the slice-to-medium concentration ratio was calculated. Each point represents the mean ± S.E.M. (n = 3–6).

saturation concentration of [^{125}I]hAβ(1–40) elimination we have reported (247 nM in injectate and 8.15 nM as cerebral concentration; Shiiki et al., 2004). The microinjected [^{125}I]hAβ(1–40) was eliminated from the male and female rat brain in a time-dependent manner up to 60 min as reported previously in male rats (Shiiki et al., 2004). The apparent elimination rate constants ($K_{app,el}$) of [^{125}I]hAβ(1–40) in male and female rats were determined to be $1.58 \times 10^{-2} \pm 0.14 \times 10^{-2}$ and $2.05 \times 10^{-2} \pm 0.15 \times 10^{-2}$ min^{-1} (mean ± S.D., n = 4–5), respectively. The value obtained for the male brains was almost identical to that reported previously (Shiiki et al., 2004).

The distribution volume of [^{125}I]hAβ(1–40) in the brain (V_{brain}) was determined by the in vitro brain slice uptake study. Fig. 1 shows the time profile of the slice-to-medium (S/M) concentration ratio of [^{125}I]hAβ(1–40) (1.8 pM). No significant difference in the S/M ratio between the 60 and 90 min incubation was observed, giving a steady-state S/M ratio of 0.694 ± 0.044 mL/g brain for males and 0.680 ± 0.056 mL/g brain for female. Incorporating $K_{app,el}$ and V_{brain} into Eq. (3), the apparent brain-to-blood hAβ(1–40) efflux clearance was determined to be 11.0 ± 3.4 μL/(min g brain) for males and 13.9 ± 3.8 μL/(min g brain) for females (mean ± S.D.). No significant differences were observed between the 7- and 8-week-old male and female rats.

3.2. Effects of hAβ peptides and fragments on [^{125}I]hAβ(1–40) elimination from rat brain

The effects of hAβ peptides and fragments were examined by both co-administration (Table 1A) and pre-administration studies (Table 1B). Little or no oligomerized [^{125}I]hAβ(1–40) was observed following the addition of 20 μM unlabeled hAβ peptides or fragments to the injection solution (Tamaki et al., 2006). [^{125}I]hAβ(1–40) (18.2 nM in injectate) elimination was significantly inhibited by the co-administration of unlabeled hAβ(1–40) (78.8% inhibition), or pre-administration of unlabeled hAβ(1–40) (79.1% inhibition) and hAβ(1–42) (36.4% inhibition). Neither co-administration nor pre-administration of hAβ(40–1), a reverse sequence analogue of hAβ(1–40), significantly affected [^{125}I]hAβ(1–40) elimination. Co-administration of hAβ(17–40) significantly enhanced [^{125}I]hAβ(1–40) elimination by 116%. Co-administration of hAβ(1–16) also enhanced [^{125}I]hAβ(1–40) elimination (114%), but this was not statistically significant. In contrast, neither co-administration of hAβ(1–42), hAβ(1–28) or hAβ(29–40) nor pre-administration of hAβ(1–43) had any effect on [^{125}I]hAβ(1–40) elimination.

3.3. Effect of P-gp and LRP-1 inhibitors on [^{125}I]hAβ(1–40) elimination from rat brain

Pre-administration of quinidine (5 mM) and verapamil (4 mM), which are P-gp inhibitors, did not significantly affect [^{125}I]hAβ(1–40) (18.2 nM in injectate) elimination, while human RAP (5 μM), an LRP antagonist, reduced [^{125}I]hAβ(1–40) elimination by 20.3% (Table 2).

Table 2
The inhibitory effect of several compounds on the elimination of [^{125}I]hAβ(1–40) from rat brain

Inhibitors	Concentration in injectate	No. of studied	BEI (%)	% of control
Control		5	60.0 ± 3.8	100
Quinidine	5 mM	4	62.3 ± 2.4	104
Verapamil	4 mM	5	65.0 ± 1.2	108
Human RAP	5 μM	5	47.8 ± 1.4**	79.7

Inhibitor solutions (50 μL) were pre-administered 5 min before administration of [^{125}I]hA((1–40) (0.02 μCi) and [^{3}H]dextran (0.20 μCi) into the same brain region. [^{125}I]hAβ(1–40) was used at a concentration of 18.2 nM (i.e. 0.6 nM as the cerebral concentration). The value was determined 60 min after intracerebral administration. Each value represents the mean ± S.E.M. $^{**}p < 0.01$, significantly different from control.

3.4. Expression of LRP-1 in rat brain and brain capillaries

The expression of LRP-1 in rat brain and rat brain capillary-rich fractions was determined by Western blot analysis using anti-LRP-1 antibody (Fig. 2). This antibody reacts with the β-chain of LRP-1. Single bands at 70 and 75 kDa were detected in crude membrane fractions of rat brain and rat brain capillary-rich fractions with the anti-LRP-1 antibody, respectively. The intensity of the single band in the crude membrane fraction of rat brain was almost as same as that found in the rat brain capillary-rich fraction. P-gp, which is selectively expressed on

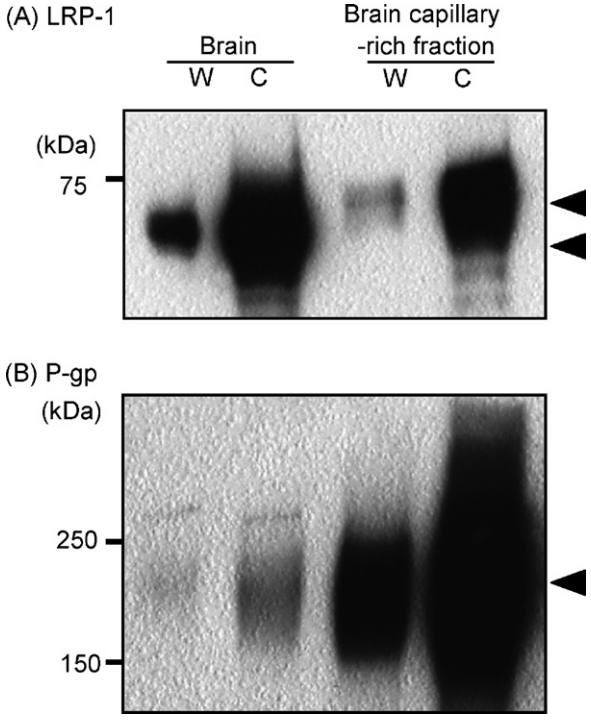

Fig. 2. Western blot analysis of LRP-1 (A) and P-gp (B) in rat brain and brain capillary-rich fractions. The whole cell lysate (W) and crude membrane fraction (C) proteins (20 μg/lane) were subjected to Western blot analysis using either anti-LRP-1 monoclonal antibody (A) or anti-P-gp monoclonal antibody (B). The molecular weight markers are shown on the left. The bands detected at 70 and 75 kDa (A) and 170 kDa (B) are indicated by arrowheads on the right-hand side.

the luminal membrane of brain capillary endothelial cells, gave an intense band at 170 kDa in the whole cell lysate and membrane fraction of the brain capillary-rich fraction compared with those of the whole brain, suggesting that the brain capillaries were concentrated in the brain capillary-rich fraction. Furthermore, in the membrane fraction of the brain capillary-rich fraction, a more intense band was detected than that in the whole cell lysate.

4. Discussion

The present study shows, firstly, that the apparent brain-to-blood efflux clearance of hAβ(1–40) from the male rat cortex is 11.0 μL/(min g brain) using a combination of BEI and brain slice uptake studies. The elimination of hAβ(1–40) from rat brain consists of partial LRP-1-mediated transport and P-gp does not significantly contribute to the elimination.

The microinjected [^{125}I]hAβ(1–40) was eliminated from the rat brain in a time-dependent manner. In the BEI study, [^{3}H]mannitol (MW 182), [^{14}C]inulin (MW 5000–5500) and [^{3}H]dextran (MW 70,000) remaining in the brain were unchanged over a 60 min period following microinjection (Zhang and Pardridge, 2001; Mori et al., 2004), suggesting that the microinjection procedure did not cause any significant disintegrity of the BBB, and leakage of the reference compound from the brain was minimal. Furthermore, the elimination of [^{125}I]hAβ(1–40) was reduced by the unlabeled hAβ(1–40) by up to 20.9% (Table 1B), indicating that at least 80% of the injected [^{125}I]hAβ(1–40) was eliminated from the brain by a saturable process at the BBB, but not by passive diffusion across the BBB.

The apparent blood-to-brain influx clearance of hAβ(1–40) has been reported to range from 0.654 to 2.43 μL/(min g brain) in the 24-week-old S.D. rat cortex in an i.v. bolus injection study (Wengenack et al., 2000; Poduslo et al., 2001). The present study determined the efflux clearance using 7–8-week-old S.D. rats, and the possibility that clearance values changed depending on age could not be ruled out. Nevertheless, since the apparent blood-to-brain influx clearance of hAβ(1–40) in the cortex was not significantly different between 8 and 24 weeks old mice (Kandimalla et al., 2005), the efflux clearance obtained in this study could be compared with the reported influx clearance. The efflux clearances determined in this study were 4.53–16.8-fold greater than these influx clearances. When the apparent efflux and influx rates were estimated using the efflux and influx clearances multiplied by the steady-state hAβ concentration in brain interstitial fluid (1.05 ng/mL) and plasma (176 pg/mL) reported in 3-month-old PDAPP mice (DeMattos et al., 2002; Cirrito et al., 2003), the efflux rate was 26.9–99.0 times greater than the influx rate (11.5 pg/(min g brain) versus 0.116–0.428 pg/(min g brain)). These results suggest that the net flux of hAβ(1–40) transport across the BBB is from brain to blood in order to eliminate Aβ from the brain.

The pre-administered hAβ(1–42) inhibited the elimination of hAβ(1–40) (Table 1B) and this suggests that the processes involved in the elimination of hAβ(1–40) also recognize hAβ(1–42). The affinity of hAβ(1–42) for the elimination process could be lower than that of hAβ(1–40), since the inhibitory effect of hAβ(1–42) was lower than that of unlabelled hAβ(1–40) (Table 1B). Therefore, the brain-to-blood hAβ transport at the BBB could be an important process in preventing the accumulation of Aβ in the brain. The co-administration of hAβ(1–42) did not significantly reduce the elimination at a higher concentration in injectate (20 μM) compared with the case of pre-administration (1 μM) (Table 1). Since the injectate was diluted 30.3-fold in the brain after co-administration (Kakee et al., 1996), the estimated concentration of hAβ(1–42) at the site of injection was calculated to be 0.66 μM. In the pre-administration study, the dilution effect of the pre-injected inhibitor solution was minimal. Therefore, the absence of significant inhibition by co-administration of hAβ(1–42) could be explained by the lower concentration of hAβ(1–42) at the site of injection compared with that in the pre-administration study.

The present study shows that the apparent brain-to-blood [^{125}I]hAβ(1–40) efflux clearance is similar in 7–8 weeks old male and female rats (Fig. 1). Epidemiological studies have suggested a higher prevalence of AD in women (Fratiglioni et al., 1997; Seeman, 1997; Andersen et al., 1999). Higher brain Aβ levels and amyloid plaques in female mice were also found in several strains of AD model mice, such as APP transgenic mice (Callahan et al., 2001; Bayer et al., 2003; Schuessel et al., 2005) and APP and presenilin-1 double transgenic mice (Wang et al., 2003). The present results indicate that a higher prevalence of AD in women is not likely to be due to the effect of gender as far as the efflux clearance of hAβ(1–40) across the BBB is concerned, while a gender difference in the elimination in aged rats remains to be investigated.

LRP has been linked to an increased risk of late-onset AD (Kang et al., 1997). Amyloid deposition in APP transgenic/RAP knockout mice was increased compared with that in APP transgenic mice without changing the APP level in the brain (Van Uden et al., 2002). Deane et al. (2004) has reported that, in RAP knockout mice, the expression level of LRP-1 and other members of the LDL receptor family was reduced in the brain and [^{125}I]hAβ(1–40) elimination from the brain was also reduced. These reports support the involvement of LRP-1 in the hAβ(1–40) elimination from the brain. Shibata et al. (2000) reported that LRP-1 is mainly involved in hAβ(1–40) efflux transport at the mouse BBB based on the inhibitory effects of human RAP and anti-LRP-1 antibody. Our previous study suggested that rat RAP did not significantly change the elimination of [^{125}I]hAβ(1–40) from rat brain (Shiiki et al., 2004). In the present study, the contribution of LRP-1 to [^{125}I]hAβ(1–40) elimination from the rat brain was assessed using human RAP, which completely inhibited rat LRP-1-mediated [^{125}I]hAβ(1–40) uptake by rat hepatocyte in vivo (Tamaki et al., 2006). Human RAP partially inhibited the elimination of hAβ(1–40) from rat brain (by 20.3%) (Table 2), yet the inhibitory effect of human RAP in rats was smaller than that reported in mice (by 44.0%) (Shibata et al., 2000). Western blot analysis suggested that LRP-1 is expressed at the rat BBB, as the LRP-1 band

was detected in rat brain capillary endothelial cells along with P-gp, which is selectively expressed in brain capillary endothelial cells (Fig. 2). Although the size detected in the brain and brain capillary-rich fractions was about 10–15 kDa smaller than that in rat liver (Field and Gibbons, 2000), a recent report showed that the molecular weight of LRP-1 in mouse brain is smaller than that in the liver due to a difference in glycosylation (May et al., 2003). These results suggest that LRP-1 is partly involved in the elimination of hAβ(1–40) in the rat brain (20.3%). This result also indicates a difference in the inhibitory effect of human and rat RAP on hAβ(1–40) elimination from rat brain. Previously, we used recombinant his- and c-myc- tagged rat RAP to examine the inhibitory effect of hAβ(1–40) elimination from rat brain. It is reported that glutathione *S*-transferase-fused RAP had an up to a 10-fold lower affinity for LRP than RAP alone (Warshawsky et al., 1993). Thus, his- and c-myc-tagged rat RAP can be considered to have a lower affinity for LRP than human RAP.

A recent study reported that the elimination of microinjected hAβ(1–40) from P-gp knockout mice brain was significantly reduced compared with age-matched wild type mice (Cirrito et al., 2005). However, LRP-1 expression in the brain capillaries of P-gp knockout mice was suppressed by 51% compared with that in wild type mice (Cirrito et al., 2005) and suppression of LRP-1 expression should attenuate the elimination of hAβ(1–40). Furthermore, unexpected gene modulation and alteration of systemic hAβ clearance in the knockout mice also cannot be ruled out. Therefore, an inhibition study involving pre-administration of quinidine or verapamil at a concentration, which completely inhibits P-gp-mediated [^{3}H]quinidine elimination from the rat brain (Kusuhara et al., 1997), was conducted. As shown in Table 2, neither quinidine nor verapamil showed any significant inhibitory effect on hAβ(1–40) elimination. This result suggests that P-gp do not make a significant contribution to the brain-to-blood efflux transport of hAβ(1–40) from the brain. Therefore, the suppression of P-gp expression and/or function would not directly affect hAβ(1–40) elimination from the brain.

Our present study suggests that the unidentified hAβ(1–40) elimination process(es) from the brain mainly contribute to the hAβ(1–40) elimination from rat brain and that the apparent brain-to-blood hAβ(1–40) efflux transport at the rat BBB is mediated by a sequence-dependent process (Table 1). To evaluate the molecules involved in the elimination of hAβ(1–40), the inhibitory effects of different hAβ fragments were examined as shown in Table 1A. Previous reports suggest that the hAβ binding site of the α5β1 integrin, the serpin-enzyme complex receptor and the insulin receptor involves the residues 5–8, 31–35 and 16–25 of hAβ, respectively (Joslin et al., 1991; Kurochkin, 1998; Matter et al., 1998; Xie et al., 2002). hAβ(1–16), hAβ(1–28), hAβ(17–40) and hAβ(29–40) tested in Table 1A were able to bind to at least one of these receptors on brain parenchymal cells, whereas these fragments did not inhibit the elimination of hAβ(1–40) from rat brain. One possible explanation for failing to observe an inhibitory effect by hAβ fragments is that multiple receptors are involved in hAβ(1–40) elimination across the BBB and these compensate for each other.

The elimination of [^{125}I]hAβ(1–40) from rat brain was significantly facilitated in the presence of hAβ(17–40), and hAβ(1–16) also had a tendency to facilitate the elimination of [^{125}I]hAβ (1–40) (Table 1A). This result implies that specific kinds of proteolytic fragments of Aβ facilitate the elimination of hAβ(1–40) from the brain across the BBB, since hAβ(1–16) and hAβ(17–40) have been reported to be major proteolytic fragments of hAβ(1–40) generated by the action of ECE, which is one of the Aβ degrading enzymes (Eckman et al., 2001). Moreover, in our previous reports, thiorphan, a NEP inhibitor, and bacitracin, an IDE inhibitor, partially reduced the apparent [^{125}I]hAβ(1–40) elimination from rat brain (Shiiki et al., 2004). Therefore, it is plausible that the brain-to-blood efflux transport of [^{125}I]hAβ(1–40) may be associated with the Aβ degradation system in the brain. A possible explanation for the facilitation of the hAβ(1–40) elimination would be that particular fragments of Aβ prevent hAβ(1–40) from binding to some binding proteins in the brain parenchyma and/or that the fragments affect the receptor for the efflux transport system of hAβ(1–40) at the BBB to increase the affinity or transport rate.

In conclusion, the present study demonstrates that LRP-1 partially affect the cerebral elimination of hAβ(1–40) and P-gp does not play a significant role in the brain-to-blood efflux transport of hAβ(1–40) at the BBB in rats. These findings contribute to our understanding of the physiological role of hAβ(1–40) efflux transport at the BBB and suggest that, as yet, unidentified molecules are involved in the cerebral hAβ(1–40) elimination process in rats.

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