Human Blood–Brain Barrier Receptors for Alzheimer’s Amyloid-β 1–40

Asymmetrical Binding, Endocytosis, and Transcytosis at the Apical Side of Brain Microvascular Endothelial Cell Monolayer

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Abstract

A soluble monomeric form of Alzheimer’s amyloid-β (1–40) peptide (sAβ1–40) is present in the circulation and could contribute to neurotoxicity if it crosses the brain capillary endothelium, which comprises the blood–brain barrier (BBB) in vivo. This study characterizes endothelial binding and transcytosis of a synthetic peptide homologous to human sAβ1–40 using an in vitro model of human BBB. 125I-sAβ1–40 binding to the brain microvascular endothelial cell monolayer was time dependent, polarized to the apical side, and saturable with high- and low-affinity dissociation constants of 7.8±1.2 and 52.8±6.2 nM, respectively. Binding of 125I-sAβ1–40 was inhibited by anti-RAGE (receptor for advanced glycation end products) antibody (63%) and by acetylated low density lipoproteins (33%). Consistent with these data, transfected cultured cells overexpressing RAGE or macrophage scavenger receptor (SR), type A, displayed binding and internalization of 125I-sAβ1–40. The internalized peptide remains intact >94%. Transcytosis of 125I-sAβ1–40 was time and temperature dependent, asymmetrical from the apical to basolateral side, saturable with a Michaelis constant of 45±9 nM, and partially sensitive to RAGE blockade (36%) but not to SR blockade. We conclude that RAGE and SR mediate binding of sAβ1–40 at the apical side of human BBB, and that RAGE is also involved in sAβ1–40 transcytosis. (J. Clin. Invest. 1998, 102:734–743.) Key words: Aβ-peptide • cerebrovascular • transporter • central nervous system • amyloidosis

Introduction

Amyloid β (Aβ),1 a heterogenous, 39–43 amino acid peptide, is the main constituent of the senile plaques and cerebrovascular deposits, the primary lesions in Alzheimer’s disease (AD) (1, 2). Aβ is considered to be central to the pathology of AD because it may be neurotoxic—directly by inducing oxidant stress and indirectly by activating microglia (3–5). It has been suggested that the presence of pathological “chaperone” proteins in brain and/or other predisposing factors is critical for the cytotoxic effects of Aβ (1). A soluble monomeric form of Aβ (sAβ) is normally produced by different types of cells (6) and is present in the circulation and cerebrospinal fluid (CSF) (7, 8) and in brain parenchyma (9). The 40-amino-acid peptide Aβ1–40 accounts for approximately 90% of Aβ released from cells in culture (6) and is the predominant form of cerebrovascular amyloid (10, 11). The longer 42-residue peptide, Aβ1–42, accounts for only 10% of secreted Aβ but is the major constituent of senile plaques (12). Recent quantitative analysis has demonstrated that 31% of all plaques contain Aβ1–40, while 60% are labeled with Aβ1–42 (13).

The origin of the Aβ deposited in cerebral vasculature and brain is uncertain. According to the “neuronal theory” Aβ is produced locally in brain. In contrast, the “vascular theory” proposes that Aβ originates from the circulation, and that circulating sAβ could contribute to neurotoxicity if it crosses the brain capillary endothelium, which comprises the blood–brain barrier (BBB) in vivo (14). Transport of several peptides and proteins through the brain capillary endothelium is possible via receptor-mediated transcytosis (15–19). It has been suggested that specific receptor mediates BBB transport of circulating sAβ1–40 in rodents and nonhuman primates (14, 20–22). However, direct transport of sAβ1–40 across the BBB in humans has not been studied, and it is not known whether specific binding and/or transport mechanisms for sAβ1–40 exist at human brain microvascular endothelial cells (HBMECs). In addition, previously used in vivo techniques (14, 21–23) lack the resolution to compare peptide binding and transport at the apical versus basolateral side of brain endothelium. We hypothesized that specific receptor(s) for sAβ1–40 are present in human brain capillaries, and that their endothelial cellular distribution may favor asymmetrical binding and transport of peptide at the BBB. Thus, apical-to-basolateral transport may allow circulating peptide to enter the brain and vessel wall.

1. Abbreviations used in this paper: Aβ, amyloid; Aβ1–40, amyloid-β (1-40) peptide; Aβ1–42, amyloid-β (1-42) peptide; AβPP, amyloid precursor protein; AβPP deposit, Alzheimer’s disease; AD, Alzheimer’s disease; Bmax, binding maximum; BBB, blood–brain barrier; CSF, cerebrospinal fluid; dIC50, dissociation constant; EPC, endothelial progenitor cell; ELISA, enzyme-linked immunosorbent assay; EPR, electron paramagnetic resonance; FCS, fetal calf serum; HBMEC, human brain microvascular endothelial cell; HPLC, high pressure liquid chromatography; Kd, dissociation constant; PAO, phenylarsine oxide; RAGE, receptor for advanced glycation end products; SR, scavenger receptor; sAβ, soluble amyloid-β; USM, University of Southern Medical School; VMEC, vascular microvascular endothelial cell; VSMC, vascular smooth muscle cell; WGA, wheat germ agglutinin; WH, wild type.
which could be associated with its cytotoxic effects. In contrast, basolateral-to-apical transport may favor clearance of s\(\beta\) from brain into blood that may ultimately decrease its tissue levels. An in vitro model of human BBB (24, 25) was used to test the above hypothesis and to characterize apical versus basolateral HBMEC binding, endocytosis, and transcytosis of a synthetic peptide homologous to human s\(\beta_{1–40}\). The receptor for advanced glycation end products (RAGE) (4) and scavenger receptor (SR) (5), were studied.

Methods

**Chemicals.** Peptide DAEFRHDSGYEVHHQKLFRVFAEDVGSNK-GAILLVMGGVV (s\(\beta_{1–40}\)), homologous to residues 672–711 of \(\beta\)-precursor amyloid protein (1–770) was custom synthesized at the W.M. Keck Facility at Yale University using solid-phase N-tert-butyl-oxy-carbonyl chemistry. Peptide was purified by reverse-phase high pressure liquid chromatography (HPLC) using a C18 column (Vydac, Hesperia, CA) and a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid (TFA). The final product was lyophilized and characterized by analytical reverse phase HPLC, amino acid analysis, and laser desorption spectrometry. The secondary structure of s\(\beta_{1–40}\) was determined by circular dichroism and consisted of 0% a-helix, 27.7% \(\beta\)-sheet, 6.0% \(\beta\)-turn, and 71.7% random coil. The reverse synthetic peptide VVGVMHLHAGKNSGVDEAFFLKVQHIVED-G5HDFRFEAD (\(\beta_{40–45}\)) was purchased from Sigma Chemical Co. (St. Louis, MO). s\(\beta_{1–40}\) was labeled with Na\(^{125}\)I (13.7 mCi/\(\mu\)g; Amersham Corp., Arlington Heights, IL) using Iodobeads (Pierce Chemical Co., Rockford, IL) following the manufacturer’s instructions. To revert the potential s\(\beta_{1–40}\) oxidation at Met35 due to the radioiodination, the peptide was subjected to reduction with 0.1 M dithiothreitol for 6 h before purification. Labeled peptides were separated from free iodine and oxidation derivatives by reverse-phase HPLC using a 5-\(\mu\)m C4 narrow bore column (Vydac) and a 45-min linear gradient from 0–70% acetonitrile. Protein elution was monitored by absorbance at 280 nm and radioactivity evaluated in a gamma counter (Beckman Instruments, Fullerton, CA). The resulting specific activity of \(^{125}\)I\(\beta_{1–40}\) was 7.6 \(\mu\)Ci/\(\mu\)g, and >97% TCA precipitable. Changes in the secondary structure due to the radioiodination procedure were evaluated by circular dichroism. Spectra in the far ultraviolet range (260–190 nm) were recorded at 24°C with a spectropolarimeter (model J-720; Jasco Inc., Easton, MD) using a cell path of 0.1 cm at a peptide concentration of 0.15 mg/ml in 20 mM Tris, pH 7.4.

**Isolation of human brain capillaries.** Human brain capillaries were isolated from small fragments of cerebral cortex obtained from surgical resections from three adults with seizure disorder, as we recently described (24). Portions with no pathologic lesions were used. Briefly, brain specimens devoid of large blood vessels were homogenized in DMEM containing 2% fetal bovine serum (FBS) using a Dounce homogenizer (VWR, San Diego, CA). The homogenate was centrifuged in 15% dextran in DMEM for 10 min at 10,000 g. The pellets containing crude microvessels were further digested in a solution containing 1 mg/ml collagenase/dispose in DMEM-S for 1 h at 37°C. Microvascular capillaries were isolated by absorption to a column of glass beads and washing off the beads. Cell viability was >95% as judged by a trypan blue exclusion test.

**Cultures and characterization of HBMECs.** The human brain microvessels were plated on rat-tail collagen-coated dishes or glass coverslips and cultured in RPMI 1640-based medium with growth factors (24). FBS, NuSerum, endothelial cell growth supplement, heparin (5 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids, vitamins, penicillin, and streptomycin (100 U/ml) (24). Cultures were incubated at 37°C in a humid atmosphere of 5% CO\(_2\). The resulting HBMECs were positive for factor VIII-related antigen, carbonic anhydrase IV, Ulex European Agglutinin I, and took up acetylated low density lipoprotein (AcLDL, ligand for endothelial SR [5]) demonstrating their endothelial origin. HBMECs also expressed \(\gamma\)-glutamyl transpeptidase, indicating their brain origin. Detailed procedures regarding characterization of HBMECs were as we previously reported (24).

In a separate set of experiments, cytoxin slides of HBMECs were prepared with a cytoxin centrifuge (Shandon Lipshaw Corp., Pittsburgh, PA), and slides were rehydrated and stained with polyclonal rabbit antihuman anti-RAGE antibodies (4). Secondary peroxidase-labeled antibodies and development with diamobenzidine were used. Sections were lightly stained with hematoxylin. Fluorescent-labeled Dil-AcLDL was used to demonstrate the presence of SR (5). HBMECs were incubated with Dil-AcLDL (10 \(\mu\)g/ml) for 4 h at 37°C, fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS), and viewed. Pictures were taken with a fluorescence microscope (Nikon, Tokyo, Japan) as described (24).

**In vitro model of the BBB.** Using the HBMECs, the BBB in vitro model was constructed in inserts with collagen-fibronectin–coated polycarbonate in Transwell membrane filters (Costar Corp., Cambridge, MA) as we reported (25). A single monolayer of HBMECs was produced by placing 200 \(\mu\)l of cell suspension containing 5 \(\times\) 10\(^5\) cells in the upper chamber of a 24-well tissue culture insert and 1,000 \(\mu\)l in the lower chamber. Analysis of the BBB by transmission electron microscopy (TEM) in 5-day-old primary cultures revealed flat monolayers of endothelial cells covering the porous membrane completely. The apical surface of endothelial cells had neither microvilli nor cytoplasmic processes. The cytoplasm of HBMECs contained elongated nuclei, free ribosomes, few mitochondria, and endoplasmic reticulum. Typical rod-shaped Weibel Palade bodies were observed with a dark striated appearance. Bundles of intermediate filaments were located under the basolateral cytoplasmic membrane, and microtubules were evenly distributed in the HBMEC cytoplasm. Occasionally, two or three HBMECs were overlaid on one another. Formation of gap and tight junctions were found between the thin cytoplasmic processes of HBMECs. As we reported, the presence of astrocytes did not positively affect the tightness of the HBMEC monolayer in this model (26), and therefore they were not used in the present studies.

s\(\beta_{1–40}\) binding to HBMEC monolayers. The HBMECs were preincubated for 1 h in RPMI culture medium with 10% FBS. The tracer solution containing \(^{125}\)I\(\beta_{1–40}\) (0.5 nM) and [\(^{14}\)C]insulin (10 nM) was added to either the apical (upper) or basolateral (lower) chamber for different periods of time within 60 min at either 37°C or 4°C. The effects of internalization (endocytosis) and metabolism of ligand by HBMECs were studied at 37°C. The inhibition of cell surface binding for Scatchard analysis was carried out at 4°C, and the tracer solution was supplemented with various concentrations of unlabeled s\(\beta_{1–40}\) (0.5–150 nM) or 150 nM s\(\beta_{40–45}\). To determine intracellular and surface-bound s\(\beta_{1–40}\), HBMEC monolayers were first washed three times in Hank’s balanced salt solution to remove unbound tracer, and then exposed to 1 ml of acid wash solution (0.2 M acetic acid and 0.5 M NaCl, pH 3.0, for 6 min at 4°C [27]). To determine which of the s\(\beta_{1–40}\) receptors participate in binding, the HBMEC monolayers were treated for 30 min at 4°C before and during \(^{125}\)I\(\beta_{1–40}\) incubation with different concentrations of anti-RAGE antibodies (4) or AcLDL, a ligand for SR (5). To block the internalization of s\(\beta_{1–40}\), HBMEC monolayers were treated for 30 min at 37°C before and during \(^{125}\)I\(\beta_{1–40}\) incubation with endocytic inhibitor, phenylarsine oxide (PAO) at 20 \(\mu\)M (28–31). Although the exact molecular mechanism for the inhibitory effect of PAO on endocytosis is not known, it has been suggested that this potent arsenical forms a stable ring with vicinal sulfhydryl groups, affects phosphoinositide metabolism, and at higher concentrations reduces cellular energy stores, but at a concentration used to inhibit endocytosis its other cellular actions are minimized (28, 29). PAO inhibits internalization of several peptide ligands (e.g., epidermal growth factor, insulin) (29), and at the BBB receptor-mediated endocytosis of insulin (30) as well as endothelial endocytosis of dynorphins (31).
The HBMECs were detached from the membrane insert using 0.1% EDTA in Hank’s solution. For degradation studies, the HBMECs were incubated with 

\[ ^{125}\text{I}\text{SA}_{1-40} \]

(3.5 nM) within 30 min at 37°C. Aliquots of the assay buffer and endothelial cell homogenates were prepared for analysis of total and TCA (12%)-precipitable \[ ^{14}\text{C} \]

radioactivities to determine the fraction of \[ ^{125}\text{I}\text{SA}_{1-40} \] that was not degraded. Uptake and metabolism of \text{SA}_{1-40}\) by HBMECs were also studied in several assay buffer and HBMEC samples with HPLC to corroborate the results of TCA precipitation analysis. Prior to extraction and HPLC fractionation, the HBMECs were exposed to acid wash solution (0.2 M acetic acid and 0.5 M NaCl for 1 min) to remove surface-bound \text{SA}_{1-40}. HBMEC samples were then prepared by hypo-osmotic lysis as reported (32). Briefly, HBMECs were homogenized in water containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 mM p-aminobenzamidine) and centrifuged at 100,000 g for 1 h at 4°C. The supernatant was then lyophilized. The resulting material was dissolved in 0.1% TFA in water, pH 2.1, before injection onto the HPLC column. The aliquots of assay buffer samples from upper and lower chamber were diluted in 0.1% TFA in water, pH 2.1, before injection onto the HPLC column. Separation was achieved using a C4 column (Vydac, The Separation Group) with a 30-min linear gradient of 25–83% acetonitrile in 0.1% formic acid over 10 min (5 min/wash) at room temperature, and then binding buffer (minimal essential medium containing fatty acid-free bovine serum albumin 0.1%) was added (50 μl/well). For radioligand binding studies, cultures revealed a flat and continuous monolayer of cells bound to the tight junctions (Fig. 1A). At 37°C, the specific binding of \text{SA}_{1-40}\) was found only at the apical side and was absent as reported (16). The inhibition of \text{SA}_{1-40}\) transport in apical-to-basolateral direction was studied in the presence of unlabeled \text{SA}_{1-40} \) (0.5–150 nM), 150 nM \text{SA}_{1-40}, and different concentrations of anti-RAGE antibody (5–40 μg/ml) and AcLDL (50–800 μg/ml). The transendothelial apical-to-basolateral influx, \text{SA}_{1-40}\) in the presence of various concentrations of unlabeled peptide in the upper chamber, \text{C}, was calculated as (16, 19): \text{SA}_{1-40} = PS × C × q, where q is the amount of protein (in mg) per number of cells in HBMEC monolayers occupying the surface area \text{A} available for transendothelial transport. Kinetic transport Michaelis–Menten parameters, \text{Km} (Michaelis affinity constant) and \text{V}_{\text{max}} (maximal transport capacity) for intact \text{SA}_{1-40}\) were calculated with a nonlinear regression analysis using SAAM II program as reported (19).

Results

The analysis of HBMECs by TEM in 5-day-old primary cultures revealed a flat and continuous monolayer of cells bound to the tight junctions (Fig. 1A). At 37°C, the specific binding of \text{SA}_{1-40}\) was found only at the apical side and was absent
from the basolateral side (Fig. 1 B). The total binding of $^{125}$I-sAβ$_{1–40}$ (0.5 nM) to the apical cell surface was rapid and progressively increased within the first 5 min. A steady-state level was reached between 5 and 60 min. The majority of binding (75–80%) was resistant to removal by acid wash, indicating incorporation into apical membrane or endocytosis at physiological temperature. The temperature dependence of sAβ$_{1–40}$ binding and internalization was evident (Fig. 1 C). At 4°C, the total binding at the apical side was significantly reduced, associated with a decrease in acid-resistant binding (i.e., intracellular binding). There was a minimal degree of metabolism or degradation of $^{125}$I-sAβ$_{1–40}$ by HBMECs at 37°C (Fig. 1 D); after 30 min and 60 min (data not shown) > 94% of peptide (corrected for the counts removed by an acid wash) remained intact in endothelial cells as confirmed by the HPLC analysis of HBMEC lysates following removal of cell surface–bound $^{125}$I-sAβ$_{1–40}$ by an acid wash. Similar results were obtained when trypsin:protease K:EDTA solution was used to remove cell surface–bound ligand (data not shown). Binding of $^{125}$I-sAβ$_{1–40}$ to the apical side was saturable in the presence of increasing concentrations of unlabeled sAβ$_{1–40}$ (Fig. 1 E, inset). Specificity of sAβ$_{1–40}$ binding was reflected by the minimal influence of 150 nM reverse sAβ$_{1–40}$ peptide. Scatchard analysis of the binding data at 4°C indicated the presence of two independent sAβ$_{1–40}$ binding sites (Fig. 1 E, main graph). The nonlinear regression analysis indicated a high-affinity binding site with a dissociation constant $K_d$ of 7.8±1.2 nM (estimate±SD) and binding maximum ($B_{max}$) of 3.75±0.52 pmol/mg of protein. The second class of binding sites was of lower affinity ($K_d$ of 52.8±6.2 nM) and higher capacity ($B_{max}$ of 15.75±0.25 pmol/mg of protein). As illustrated in Fig. 1 F, the amount of sAβ$_{1–40}$ that was internalized by HBMECs (i.e., resistant to re-

Figure 1. (A) TEM of HBMEC monolayer showing continuous endothelial cells bound to each other with tight junctional complexes (arrow); ***supporting membrane (×19,000). (B) Distribution of sAβ$_{1–40}$ receptors in HBMEC monolayers. $^{125}$I-sAβ$_{1–40}$ (0.5 nM) was added to either apical or basolateral compartment of HBMECs grown on supporting membrane. Total (solid circles) and acid-resistant (open circles) binding at 37°C at the apical side of the HBMEC. Binding of $^{125}$I-sAβ$_{1–40}$ was undetectable at the basolateral side (solid squares). (C) At 4°C, total binding (solid circles) at the apical side was significantly reduced associated with a decrease in acid-resistant binding (open circles). (D) The HPLC profile of HBMEC lysates prepared following exposure to $^{125}$I-sAβ$_{1–40}$ (3.5 nM) for 30 min at 37°C show mainly (94.1%) intact peptide in acid-resistant fraction following acid wash (main graph). An HPLC profile of intact $^{125}$I-sAβ$_{1–40}$ was shown as a control (inset). (E) Saturable binding of $^{125}$I-sAβ$_{1–40}$ to the apical surface at 4°C in the presence of increasing concentrations of sAβ$_{1–40}$ was not affected by the reverse peptide, sAβ$_{40–1}$. Scatchard analysis (main graph) showed two independent classes of binding sites. Nonlinear least squares regression analysis was used to estimate the dissociation constant ($K_d$) and binding capacity ($B_{max}$) of the high affinity site as 7.8±1.2 nM and 3.75±0.52 pmol/mg of protein (estimate±SD). For the low-affinity, high-capacity sites, $K_d$ was estimated at 52.8±6.2 nM, and $B_{max}$ = 15.75±0.25 pmol/mg of protein. (F) Effect of 20 μM PAO on the internalization of $^{125}$I-sAβ$_{1–40}$. The total amount of $^{125}$I-sAβ$_{1–40}$ bound to the HBMECs, as well as the extracellular (acid wash removable) and intracellular (acid wash resistant) components were examined following a 30-min incubation at 37°C. *P < 0.05, for total and acid resistant $^{125}$I-sAβ$_{1–40}$ nonsignificant for acid removable $^{125}$I-sAβ$_{1–40}$ in PAO-treated compared with respective controls, by Student’s t test. (B, C, E, and F) The values are mean±SD of three monolayers determined in triplicate; $mg_p$ = mg of protein.
removal by acid wash) was significantly reduced by 20 μM PAO, an endocytic inhibitor. In all experiments, the HBMEC uptake of simultaneously exposed 14C-inulin was barely above the background level, indicating the absence of a nonspecific leak into the cells and minimal entrapping of the tracer in the medium between the cells at the sites of junctional complexes.

Transendothelial transport of sAβ1-40 was asymmetrical in the apical-to-basolateral direction. At 37°C, transport from the upper to the lower chamber was significantly higher for intact 125I-sAβ1-40 (TCA-precipitable) than for 14C-inulin, an extracellular space marker transported across the monolayer by a nonspecific diffusion (leakage) through the intercellular pores or junctions (Fig. 2 A). In contrast, basolateral-to-apical transport of sAβ1-40 was comparable to inulin. Apical-to-basolateral transport of sAβ1-40 across the endothelial monolayer corrected for the nonspecific leakage (inulin transport) was time dependent and sensitive to reduced temperature (Fig. 2 B).

When corrected for the nonspecific leakage, basolateral-to-apical transport of 125I-sAβ1-40 was undetectable. Figure 2 C illustrates dose-dependent inhibition of 125I-sAβ1-40 PS product (corrected for the nonspecific leakage) by increasing concentrations of unlabeled sAβ1-40 in the apical (upper) chamber. The specificity of transport was shown by the lack of effect of sAβ1-40. The Michaelis–Menten analysis revealed the Kn of 45 ± 9 nM and Vmax of 2.10 ± 0.35 pmol/min/mg of protein (Fig. 2 D).

Figure 3 A indicates that anti-RAGE antibody (20 μg/ml) inhibits by about 63% the binding of 125I-sAβ1-40 to the apical side of the BBB. AcLDL at a maximal inhibitory concentration of 800 μg/ml was able to produce about 32% inhibition of 125I-sAβ1-40 binding, and a further increase in the AcLDL concentration did not result in a greater decrease of 125I-sAβ1-40 binding (data not shown). Maximal inhibition of 125I-sAβ1-40 binding by anti-RAGE + AcLDL was 95.6%. As shown by the inset, there was a progressive dose-dependent inhibition of 125I-sAβ1-40 binding by anti-RAGE at 4°C in the presence of a maximal inhibitory concentration of AcLDL; the maximal inhibition with anti-RAGE was achieved at 20 μg/ml. Figure 3 B shows that at 37°C, anti-RAGE (20 μg/ml) inhibits apical-to-basolateral transport of 125I-sAβ1-40 by about 36%, and further increase in the anti-RAGE concentration did not produce greater inhibition (Fig. 3 B, inset). The addition of AcLDL at maximal concentrations did not affect transcytosis of 125I-sAβ1-40.

The expression of RAGE and SR in the present HBMEC system was shown by immunocytochemistry (RAGE) and uptake of endothelial SR ligand, fluorescent Dil-AcLDL (Fig. 3, C and D). We previously reported that glycoprotein 330, gp330/megalin, is expressed at the BBB, but does not participate in binding and transport of free Aβ1-40 (38). This has been confirmed in the present BMEC model and the expression of gp330/megalin shown by immunocytochemistry with mouse monoclonal 1H12 anti–human anti-gp330 antibodies, kindly provided by R. McCluskey, Harvard University (data not shown; Stins et al., unpublished observations). Figure 3 E illustrates that unlabeled LDL (low density lipoproteins) at concentrations as high as 800 μg/ml does not affect the uptake of 125I-AcLDL (1 nM) exposed for 1 h to the apical side of the HBMEC monolayers, indicating that LDL receptor does not participate in uptake of AcLDL, as shown previously (39, 40).

Our results with cultured HBMECs led us to analyze directly the potential involvement of RAGE and SR in cellular handling of sAβ1-40 using transfected cell systems in which the

![Figure 2](http://www.jci.org)  
**Figure 2.** Asymmetrical transendothelial transport of sAβ1-40 across HBMEC monolayers. (A) 125I-sAβ1-40 (0.5 nM) and 14C-inulin (10 nM) were added to either apical or basolateral compartment of HBMEC for 30 min at 37°C. Ordinate, percent of the radioactivity dose of 125I-sAβ1-40 (open bars) and 14C-inulin (patterned bars) transported from either apical-to-basolateral (left), or basolateral-to-apical (right) compartment. *P < 0.05, ns nonsignificant for 125I-sAβ1-40 and 14C-inulin, by Student’s t test. (B) Time and temperature dependence of apical-to-basolateral 125I-sAβ1-40 transport corrected for the nonspecific leakage of tracers (solid points at 37°C; open points at 4°C). Basolateral-to-apical transport (open squares) was undetectable at 37°C. (C) Inhibition of the permeability-surface area (PS) product of 125I-sAβ1-40 (transport in apical-to-basolateral direction) by increasing concentrations of unlabeled sAβ1-40 at the apical side, and lack of the effect of sAβ1-40. (D) Apical-to-basolateral saturable influx (I<sub>max</sub>) of 125I-sAβ1-40 determined at 37°C in the presence of increasing concentrations of unlabeled sAβ1-40. (B, C, and D) The values for intact 125I-sAβ1-40 were corrected for the diffusion across intercellular junctions (nonspecific leakage of tracer) by subtracting corresponding values for 14C-inulin. Values are mean ± SD of three monolayers determined in triplicate; mg<sub>p</sub> = mg of protein.
Aβ-cellular interactions were mediated principally by the latter two receptors. CHO cells transiently transfected with pcDNA3/RAGE displayed expression of RAGE, by immunoblotting and immunocytochemistry, compared with mock-transfected controls (Fig. 4A, B1–II). Transiently transfected CHO cells demonstrated specific binding of \(^{125}\text{I}\)-sA\(_{1–40}\) compared with mock-transfected controls (Fig. 4C). Cells were briefly exposed to paraformaldehyde (2%) to prevent internalization during binding assays performed at 37°C, and RAGE-dependent \(^{125}\text{I}\)-sA\(_{1–40}\) binding was dose-dependent with \(K_d = 72.0 \pm 42.1\) nM. Cell-associated radioactivity was restricted to the surface, as shown by complete elution of specifically bound \(^{125}\text{I}\)-sA\(_{1–40}\) with heparin under these conditions (Fig. 4D, fixed cell). If heparin solution was followed by dissolution of the cells in NP-40 containing buffer, no additional specifically bound tracer was recovered using paraformaldehyde-treated cells. In contrast, when experiments were performed at 37°C without paraformaldehyde, endocytosis of cell-associated \(^{125}\text{I}\)-sA\(_{1–40}\) resulted in two pools of ligand (Fig. 4D, unfixed cell); most ligand was still on the cell surface (~82–83% by 3 h). However, a component of specifically bound ligand was present in a heparin-resistant, NP-40–elutable pool (~17–18%). Immunocytochemistry was performed using an antibody specific for A\(_{1–40}\). To visualize internalized ligand, following the incubation period at 37°C, cell surface sA\(_{1–40}\) was eluted with heparin-containing buffer. The absence of surface-associated ligand following heparin treatment of RAGE-transfected CHO cells was confirmed by immunocytochemistry of nonpermeabilized cells (Fig. 4E, I). Next, after heparin elution, cultures were permeabilized with NP-40, and the presence of a pool of sA\(_{1–40}\) present in a granular pattern was observed (Fig. 4E, II). Thus, the latter sA\(_{1–40}\) represented internalized ligand.

Similar radioligand and internalization studies were performed using stably transfected Bowes cells overexpressing SR type A. This scavenger receptor was selected for our studies in view of its identification in brain microvasculature (39). Surface binding was evaluated at 37°C using cells briefly exposed...
Compared with mock-transfected controls, SR-transfected Bowes cells displayed specific binding of 125I-sAβ1–40. Binding was dose dependent, displaying $K_d \approx 101.7 \pm 32.9$ nM (Fig. 5A). Pretreatment of cultures with paraformaldehyde prevented internalization of cell-bound 125I-sAβ1–40 as complete elution of specifically bound ligand was observed with heparin (Fig. 5B, fixed cell). Under these conditions, no increments in specifically bound 125I-sAβ1–40 was observed following NP-40 elution. In contrast, experiments with live (i.e., unfixed) cells and 125I-sAβ1–40 performed at 37°C demonstrated the appearance of a sizeable pool of heparin-resistant cell-associated tracer. Following heparin elution of cell surface–bound tracer, NP-40 elution recovered an additional $\sim 70\%$ of total specifically cell-associated radioactivity. Immunocytochemistry was used to confirm the presence of internalized Aβ. Following incubation of cultures with sAβ1–40 at 37°C, cultures were exposed to heparin to elute surface-bound ligand (Fig. 5C, I). Subsequent permeabilization of these cells with NP-40 containing buffer (EII) resulted in the appearance of a pool of sAβ1–40 in a granular pattern (Fig. 5C, II) which represented internalized ligand.

**Discussion**

These studies indicate that sAβ1–40 is bound and internalized at the apical side of the HBMECs cultured on membrane inserts.
by two specific acceptor sites. The binding constant of the high-affinity sAβ₁₋₄₀ BBB sites (K_d = 7.8 nM) was comparable to K_d′s determined for human brain capillary receptors for peptides such as leptin, transferrin, insulin, and insulin-like growth factors (41). The binding maximum of the high-affinity sAβ₁₋₄₀ BBB sites was at least an order of magnitude higher than for the peptide hormones. The second class of lower affinity sites (K_d = 52.8 nM) exhibited 4.2-fold greater binding capacity than the high-affinity sites. The inhibition studies indicated that RAGE and SR are receptors involved in binding of free sAβ₁₋₄₀ at the apical side of the BBB. In contrast, the BBB binding and transport of Aβ complexed to apolipoprotein J (apoJ) is mediated via gp330/megalin (38), and this receptor does not recognize the free form of peptide. The specific transport system mediating asymmetrical apical-to-basolateral transcytosis of free sAβ₁₋₄₀ was also found. The affinity constant of human BBB sAβ₁₋₄₀ transporters (45 nM) compares well with the initially reported K_d of 42 nM for saturable sAβ₁₋₄₀ BBB transport in vivo in rodents (20). Transport was partially sensitive to RAGE blockade but also involved a RAGE-independent component, possibly a novel cellular “chaperone” transporter protein for sAβ.

The lack of specific sAβ₁₋₄₀ binding at the basolateral side of the HBMEC monolayer suggests that all specific high-affinity binding sites at normal human BBB may be located at the apical side, similar as for insulin (30). The polarity of the BBB in present HBMEC model (24, 25) seems to be preserved (42), as indicated by cellular distribution of the A amino acid transporter and enrichment of the sodium pump at the basolateral side (Stins, M., S. Jovanovic, J.B. Mackic, K.S. Kim, and B.V. Zlokovic, unpublished observations). Previous work on in vitro BBB models indicated that several peptides (e.g., vasopressin, atrial natriuretic peptide) in contrast to sAβ₁₋₄₀ are taken up by specific mechanisms only at the basolateral side followed by transendothelial transport and exocytosis at the apical side, while apical-to-basolateral transport was undetectable (16). In the absence of significant brain-to-blood transport of sAβ, rapid clearance of sAβ₁₋₄₀ from the CSF, as reported in rodents (43), could be important to maintaining low concentrations of sAβ in brain extracellular fluids. It is likely that present in vitro findings could be extrapolated to humans in vivo, since the in vivo evidence in rodents and nonhuman primates confirms the net sAβ₁₋₄₀ BBB transport from blood-to-brain (20–23, 44).

In addition to clearance, it is likely that brain metabolism serves to maintain low levels of sAβ in cerebrovascular and brain interstitium to balance the effects of BBB transport and local brain production of peptide, which both would tend to accumulate sAβ in brain. In contrast to metabolism of sAβ₁₋₄₀ in brain in vivo (22, 44), there was minimal degradation of peptide by the HBMECs. The failure in degradation mechanisms combined with an enhanced apical-to-basolateral sAβ transport, as in senescent squirrel monkey (22), may enhance the development of cerebral amyloid angiopathy and increase sAβ accumulation in brain. Whether this same situation applies to BBB mechanisms in AD remains to

Figure 5. Interaction of SR with sAβ₁₋₄₀ studies on SR-transfected Bowes cells. (A) Dose-dependence of ¹²⁵I-sAβ₁₋₄₀ binding. SR-transfected Bowes cells were incubated with the indicated concentrations of ¹²⁵I-sAβ₁₋₄₀ alone or in the presence of 100-fold excess of unlabeled ligand. Specific binding was determined and analyzed as described under Fig. 4 C. (B) Identification of surface-associated and internalized ¹²⁵I-sAβ₁₋₄₀. SR-transfected Bowes cells were either briefly treated with paraformaldehyde (fixed cells) or incubated in buffer alone (unfixed cells), followed by exposure to ¹²⁵I-sAβ₁₋₄₀ as under Fig. 4 D. (C) Visualization of cell-associated sAβ₁₋₄₀. SR-transfected Bowes cells were incubated with sAβ₁₋₄₀ at 37°C for 2 h. Cells were exposed to heparin containing buffer to elute surface bound Aβ (CI), or were exposed to heparin followed by permeabilization with NP-40 containing buffer (CII). Immunocytochemistry to visualize Aβ was performed as described in the text.
be elucidated. It has been suggested that Aβ1–42 produced in brain may favor amyloid “nucleation” (45) along with other components, e.g., zinc and apoE, to accumulate soluble sAβ1–40 from blood and CSF (1, 2, 46). At critical levels (47), and in the presence of other predisposing factors (e.g., apoE4 genotype, mutations in presenilin 1 and 2 genes, and mutations in amyloid-β protein precursor gene) (1) sAβ may precipitate as amyloid fibril in the vascular wall or in brain parenchyma, could exert its cytotoxic effects (3–5) and contribute to neuronal loss and the development of AD pathology (1).

Present findings support the vascular theory by demonstrating that brain endothelial distribution of Aβ receptors at the apical side of the BBB could be responsible for interaction of circulating sAβ with the vessel wall, both for removal of this peptide from plasma and possibly for inducing endothelial cell injury (48, 49). The list of specific receptor- and/or carrier-mediated BBB mechanism(s) participating in cerebrovascular accumulation, deposition, and brain transfer of blood-borne Aβ1–40, the Dutch mutant vasculotropic peptide (AβQ) (22) and Aβ1–42 free or complexed with apoE and apoE4 (21, 32, 38), and/or mediating vascular endothelial damage by Aβ (3, 4, 48, 49) includes gp330/megalin, RAGE, SR, low- and high-density liporotein receptors, vascular adhesion molecules, endoplasmic reticulum-associated Aβ binding protein (35) and possibly yet unidentified receptors/transporters.

The BBB permeability to Aβ–apoJ complex via gp330/megalin receptor was among the highest ever measured for peptides/proteins at the BBB (38). The gp330/megalin at the BBB works with the maximal transport capacity at physiological plasma concentrations of apoJ (50), but despite its extremely high affinity for Aβ–apoJ, this receptor does not transport or bind free Aβ at the BBB (38). The present study reveals that RAGE and SR are responsible for BBB binding of free Aβ. Both the RAGE and SR are well known as receptors with multiple functions including cellular endocytosis and transcytosis of macromolecules (3, 51). Our studies support the potential of RAGE and SR type A to interact with sAβ1–40, mediating surface binding and, potentially, subsequent cellular processing of the ligand. In terms of endocytosis of cell-bound Aβ, SR type A appears to be much more efficient than RAGE. However, RAGE may function to pass off Aβ to other binding sites mediating passage through the endothelial monolayer by mechanisms not adequately modeled in our transfected cell system. Since the transfection studies were performed in CHO and Bowes cells to examine separately the contributions of RAGE and SR, respectively, rather than endothelium, quantitative comparisons of the data are difficult to make. Clearly, the crucial test of our hypothesis concerning receptor-dependent interactions of intravascular Aβ will be in vivo studies using specific reagents to block contribution individually of each receptor to cellular handling of Aβ.

With respect to Aβ BBB transcytosis, the present study suggests the involvement of RAGE but also indicates a possible role for a RAGE-independent component that may be a novel cytosolic Aβ “chaperone” transporter protein. The molecular nature of this chaperone transporter protein remains to be elucidated, as well as its functional relationship with RAGE. It is possible that both RAGE and chaperone protein exhibit similar kinetic affinities to transport Aβ in the present HBMEC model, so that Michaelis–Menten analysis revealed a single transport site. Alternatively, the affinity of one of these components may be at the lower limit of sensitivity with the present method, and molecular isolation and characterization from cytosolic and cell membrane fractions should demonstrate whether the transporter protein is different from known Aβ-binding proteins and/or receptors. The distribution of cerebrovascular endothelial receptors for free and bound forms of Aβ in AD is unknown, but it is likely that they could play important roles in Aβ vascular binding and transport, and therefore influence the development of cerebrovascular amyloidosis and vascular injury.

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