The Low Density Lipoprotein Receptor-related Protein 1 Mediates Uptake of Amyloid β Peptides in an in Vitro Model of the Blood-Brain Barrier Cells

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The metabolism of amyloid β peptide (Aβ) in the brain is crucial to the pathogenesis of Alzheimer disease. A body of evidence suggests that Aβ is actively transported from brain parenchyma to blood across the blood-brain barrier (BBB), although the precise mechanism remains unclear. To unravel the cellular and molecular mechanism of Aβ transport across the BBB, we established a new in vitro model of the initial internalization step of Aβ transport using TR-BBB cells, a conditionally immortalized endothelial cell line from rat brain. We show that TR-BBB cells rapidly internalize Aβ through a receptor-mediated mechanism. We also provide evidence that Aβ internalization is mediated by LRP1 (low density lipoprotein receptor-related protein 1), since administration of LRP1 antagonist, receptor-associated protein, neutralizing antibody, or small interference RNAs all reduced Aβ uptake. Despite the requirement of LRP1-dependent internalization, Aβ does not directly bind to LRP1 in an in vitro binding assay. Unlike TR-BBB cells, mouse embryonic fibroblasts endogenously expressing functional LRP1 and exhibiting the authentic LRP1-mediated endocytosis (e.g. of tissue plasminogen activator) did not show rapid Aβ uptake. Based on these data, we propose that the rapid LRP1-dependent internalization of Aβ occurs under the BBB-specific cellular context and that TR-BBB is a useful tool for analyzing the molecular mechanism of the rapid transport of Aβ across BBB.

Aggregation and deposition of amyloid β-peptide (Aβ) in the brain are crucial events in the pathogenesis of Alzheimer disease (AD) (1). Aβ is produced from β-amyloid precursor protein through sequential proteolytic cleavages by β- and γ-secretases. Missense mutations as well as duplication of the β-amyloid precursor protein gene have been identified in pedigrees of early onset familial AD (2–4), some of which have been shown to alter the β-amyloid precursor protein processing in such a way as to increase the level of Aβ, especially that of the more aggregable species Aβ42, leading to formation of Aβ fibrils (5–7). Cognitive deficits in transgenic mice overexpressing familial AD mutant form β-amyloid precursor protein, as well as alteration in synaptic plasticity and synapse loss induced by Aβ oligomers implicate aggregated species of Aβ in the neuronal dysfunction and death in AD brains (8–10).

Aβ, secreted from neurons in the brain, is thought to be catabolized by specific proteases (e.g. NEP and insulin-degrading enzyme) (11, 12); phagocytic cells in brains (i.e. microglia and astrocytes) (13, 14) take up and clear soluble or aggregated Aβ. Notably, in vivo observations that Aβ injected into rodent brains is rapidly effluxed from brains (15, 16) suggest the presence of a novel pathway for Aβ clearance across the blood-brain barrier (BBB). The BBB is considered as a three-cell archetype composed of brain microvascular endothelial cells (BMECs), astrocytes, and supporting pericytes. It does not normally allow a free exchange of macromolecules between brain and blood, due to the presence of tight junctions formed by BMECs (17), which suggests a receptor-mediated transport mechanism for the efflux of Aβ across the BBB. It has been suggested that LRP1 (low density lipoprotein receptor-related protein 1) is involved in the Aβ efflux transport at the BBB in vivo (15, 18), although there is no direct evidence to support the role of LRP1 in BMECs, the latter bordering the outer limit of the BBB. Thus, there is a compelling need for an endothelial cell model that can

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** The abbreviations used are: Aβ, amyloid β peptide; AD, Alzheimer disease; BBB, blood-brain barrier; BMEC, brain microvascular endothelial cell; P-gp, P-glycoprotein; PPA, tissue-type plasminogen activator; RAP, receptor-associated protein; PICUP, photo-induced cross-linking of unmodified proteins; MEF, mouse embryo fibroblast; siRNA, small interference RNA; RNAi, RNA interference; RAGE, receptor for advanced glycation end products; HUVEC, human umbilical vein endothelial cell; ANOVA, analysis of variance.
recapitulate the transport of Aβ and other macromolecules across the BBB and with which a precise molecular mechanism of Aβ transport across the BBB could be elucidated.

To verify the molecular and cellular mechanisms of Aβ transport across the BBB, application of cell biological and physiological techniques at a single cell level is mandatory. In this study, we adopted the TR-BBB cells, a conditionally immortalized cell line derived from brain capillary endothelial cells of transgenic rats expressing temperature-sensitive large T antigen (19), whose inactivation upon incubation at 37 °C renders the cells into a nonimmortalized state similar to primary BMECs. TR-BBB cells have been shown to express a number of receptors and transporters expressed in endothelial cells comprising the BBB (e.g. GLUT-1, P-glycoprotein (P-gp), and other influx or efflux transporters, allowing for the characterization of their functions in vitro (20).

Using TR-BBB cells, we established an in vitro model of Aβ uptake and found that LRP1 is involved in the rapid and robust Aβ internalization in TR-BBB cells. In contrast, fibroblasts or neuroblastoma cells that express LRP1 did not internalize Aβ, suggesting that LRP1 is not sufficient for the rapid internalization of Aβ, implicating cell type specificity in the LRP1-dependent Aβ uptake. Our observations provide a new clue to the molecular mechanism of Aβ uptake and transport across the BBB.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Synthetic Aβ-(1–40) and Aβ-(1–42) were purchased from Peptide Institute, Inc. (Osaka, Japan). 125I-radiolabeled Aβ-(1–40) (125I-Aβ) was purchased from PerkinElmer Life Sciences. Polyclonal anti-LRP1 antibody, RRR is a rabbit polyclonal antibody that recognizes full-length LRP1 (21). Anti-LRP1 monoclonal antibody 11H4 and human full-length LRP1 cDNA were provided by Dr. A. Kinoshita (22). Anti-LRP1 monoclonal antibody R488 recognizes both heavy chain and light chain of LRP1 (23). Anti-LRP1 polyclonal antibody R488 recognizes both heavy chain and light chain of LRP1 (23). Anti-LRP1 polyclonal antibody LRPI-IC was raised against C-terminus of human LRP1 (LLGRGPEDEIGDPLA). Anti-Aβ monoclonal antibody 82E1 was purchased from Immuno-Biological Laboratories Co., Ltd. LRP1 was purified from human placenta as previously described (24). tPA was purchased from American Diagnostica, Inc. (Stamford, CT). Anti-tPA antibody was purchased from Oxford Biomed Research (Oxford, MS). Anti-receptor for advanced glycation end products (RAGE) antibody and control OX-6 antibody were purchased from Oxford Biomed Research (Oxford, MS). Anti-tPA antibody was purchased from Immuno-Biological Laboratories Co., Ltd. IODO-GEN-precoated tubes and D-Salt polyacrylamide desalting columns, 6000 molecular weight cut-off were from Pierce. IODO-GEN-precoated tubes and D-Salt polyacrylamide desalting columns, 6000 molecular weight cut-off were from Pierce.

Preparation of Aβ—125I-Aβ peptides were solubilized in distilled water at a concentration of 0.1 nm and stored at −80 °C until use. Synthetic Aβ peptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (Kanto Chemical) at a concentration of 1 mg/ml, dried, and resolubilized in phosphate-buffered saline containing 3% (v/v) Me3SO (Kanto Chemical) upon use. We confirmed that 125I-Aβ migrates at 4 kDa on SDS-PAGE and does not show signs of aggregation at concentrations used in this study (see supplemental Fig. 1). In addition, synthetic Aβ utilized in this study showed no sign of fibrillization as examined by a thioflavin T fluorescence assay (data not shown).

Immunoblot Analysis—SDS-PAGE was performed as described previously (29). The immunoblots were visualized using Immuno-star reagents (Wako Pure Chemical) and visualized using LAS-1000plus (FUJIFILM) as described (29).

PICUP Experiment—0.1 nm 125I-Aβ or 20 μm of unlabeled Aβ-(1–40) was subjected to a PICUP reaction. The resulting samples were separated by SDS-PAGE using 10–20% Tris-glycine gradient gels and analyzed by immunoblotting or autoradiography as described (30, 31).

Cell Culture—TR-BBB cells were grown in collagen type I-coated 75-cm² tissue flasks (BD Biosciences) at 33 °C under 5% CO2/air. Dulbecco’s modified Eagle’s medium supplemented with 1.5 mg/ml sodium bicarbonate, 15 μg/ml bovine endothelial cell growth factor (Roche Applied Science), 70 μg/ml benzyl-penicillin potassium, 100 μg/ml streptomycin sulfate, and 10% fetal bovine serum was used as culture medium.

Mouse embryonic fibroblasts (MEFs) genetically deficient in LRP1 (PEA13 cells), LRP1 (+/−) MEFs (PEA10 cells), and normal MEFs (MEF-1 cells) derived from the same mouse strain were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, as described previously (32). Chinese hamster ovary cells (from ATCC), Neuro2a cells (from ATCC), and McARH7777 cells (provided by Dr. J. Aoki) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (from Sigma). IODO-GEN-precoated tubes and D-Salt polyacrylamide desalting columns, 6000 molecular weight cut-off were from Pierce.

In Vitro Uptake Assays of 125I-Labeled Proteins in TR-BBB Cells—The uptake of 125I-Aβ or 125I-tPA into TR-BBB cells was examined as reported previously (33). Briefly, TR-BBB cells cultured onto collagen I coated 24-well dishes were grown to 90–100% confluence. Prior to experiments, cells were washed three times with 1 ml of ECF buffer (138 mM NaCl, 5.0 mM KCl, 1.3 mM CaCl2, 0.8 mM MgCl2, 0.3 mM KH2PO4, 0.3 mM Na2HPO4, 5.6 mM D-glucose, 10 mM HEPES, pH 7.4) and incubated at 37 °C with 125I-labeled ligand proteins for a predetermined time period. After incubation, 125I-labeled ligand proteins were removed, and the cells were washed three times with 1 ml of ice-cold ECF buffer and an additional three times with...
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acid wash buffer (28 mM CH₃COONa, 120 mM NaCl, 20 mM sodium barbital, pH 3.0). In some experiments, cells were further treated with Pronase (1 mg/ml; Sigma) for 1 h at 4 °C to completely remove cell surface-attached radioligands. The amount of surface-bound labeled ligands was calculated as the amount of ligands released by this treatment, and the amount of internalized ligands was defined as the amount of ligands that remained associated with the cell pellet following this treatment. Cells were solubilized with 200 μl of 5 N NaOH for 12 h, and the protein amount in the cells was measured by the Lowry method. The amount of the cell-associated ligands was expressed as the cell/medium ratio (see below).

The cell/medium ratio (μg/mg of protein) is equal to 125I counts in the cells (cpm/mg protein)/125I counts in the incubation medium (cpm/μl). When the effects of various inhibitors or siRNA against LRP1 were studied, TR-BBB cells were incubated with 125I-Aβ and 125I-tPA for 5 and 10 min, respectively.

In the efflux assay, TR-BBB cells were first incubated with 125I-Aβ for 10 min (first incubation). After incubation, the media were replaced with the ECF buffer (incubation medium), and cells were further incubated at 37 °C for a various time period to allow efflux of Aβ (second incubation). Degradation of 125I-Aβ was studied by a trichloroacetic acid precipitation assay (15). Incubation medium was mixed with trichloroacetic acid (final concentration, 10%) and bovine serum albumin (final concentration, 1.25%) and centrifuged for 20 min, and the radioactivities in the precipitate and supernatant were quantitated by a γ counter. We confirmed that ~85–90% of total 125I-Aβ in medium prior to the first incubation was trichloroacetic acid-precipitable. Thus, we quantitated the extent of trichloroacetic acid precipitability of Aβ prior to the first incubation upon each experiment and normalized the level of trichloroacetic acid-precipitable Aβ in incubation medium at each time point upon the second incubation by the precipitation extent and indicated the ratio of trichloroacetic acid-precipitable Aβ as the percentage of that at time 0 of the first incubation. For the uptake assay of Aβ-apoE complex, Aβ was preincubated with apoE4-containing lipoproteins (2 μg/ml) for 30 min at room temperature.

To examine the involvement of heparan sulfate proteoglycans or chondroitin sulfate proteoglycans in 125I-Aβ uptake, TR-BBB cells were preincubated in medium containing heparinase I (Seikagaku), heparinase II (Sigma), heparinase III (Sigma), or chondroitinase ABC (Seikagaku) at a concentration of 1 unit/ml for 4 h at 37 °C, followed by three washes with ECF buffer.

Transfection of Small Interference RNA (siRNA) against Rat LRP1—Control siRNA (Stealth™ RNAi negative control medium GC duplex 2) and two Stealth RNAs against rat LRP1 (siRNA 1, 5′-UUGACAUUCGGAAUCAAGACAGUGGG-3′; siRNA 2, 5′-UGAUAUCCGUUUAUGGCUUGGG-3′) were purchased from Invitrogen. TR-BBB cells cultured in 24-well dishes were transfected with 42 pmol of siRNA duplex using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The Lipofectamine RNAiMAX and siRNA mixture were incubated with TR-BBB cells for 30 h. Transfected TR-BBB cells were collected for immunoblotting or uptake assays as described above.

Binding of Aβ to Immobilized LRP1 in Vitro—An in vitro binding assay was performed as described previously (29). Briefly, microtiter wells were coated with purified full-length human LRP1 or recombinant sLRP2, sLRP4, or bovine serum albumin (negative control) at 4 °C. Unoccupied sites were blocked with Block Ace (Snow Brand, Sapporo, Japan) and washed with phosphate-buffered saline containing 0.05% Tween 20. Wells were then incubated with 100 nM Aβ or tPA in the presence or absence of 1 μM RAP for 12 h, washed with phosphate-buffered saline containing 0.05% Tween 20, and then reacted with an anti-Aβ antibody (BA27; gift of Takeda Pharmaceutical Co., Ltd.) (34) or anti-tPA (Oxford Biomedical Research) antibodies for 3 h. After incubation with a horseradish peroxidase-tagged secondary antibody (GE Healthcare), the levels of ligands bound to LRP1 were quantitated by development using a TMB microwell system (KPL, Inc.).

Preparation of Radiolabeled tPA—Human tPA was iodinated, using IODO-GEN-precoated tubes, according to the manufacturer’s instructions. The unincorporated 125I was removed using a D-salt polyacrylamide desalting column.

RESULTS

Aβ Is Internalized by TR-BBB Cells in a Specific Manner—We reasoned that if Aβ is transported across the BBB, Aβ should be internalized by BMECs. To test this, we used TR-BBB cells, an in vitro cellular model in which the internalization in BBB endothelial cells can be analyzed (19). We first evaluated the uptake of 125I-Aβ in monolayer cultures of TR-BBB cells.

First, we checked whether 125I-Aβ-(1–40) does not form oligomers at the concentration used in this study (0.1 nM) by a light-induced chemical cross-linking experiment, PICUP, an efficient method for detecting SDS-sensitive Aβ assemblies by forming covalently cross-linked Aβ oligomers (30, 31). The oligomerization state of each peptide after cross-linking was determined by immunoblot analysis or autoradiography. When 20 nM of unlabeled Aβ was subjected to PICUP reaction, several covalently cross-linked oligomer bands (i.e. dimer, trimer, and tetramer) appeared in an irradiation-dependent manner, suggesting that a fraction of Aβ formed SDS-sensitive oligomers in the solution (supplemental Fig. 1). In contrast, 0.1 nM 125I-Aβ-(1–40) did not exhibit any cross-linked oligomers, and only a single band migrating at 4 kDa was detected even after the PICUP reaction (supplemental Fig. 1). This suggested that the concentration of 125I-Aβ-(1–40) used in this experiment was low enough to eliminate formation of oligomers.

Cells grown in collagen-coated dishes were incubated with 125I-Aβ-(1–40). After incubation, cells were extensively washed with acid wash buffer to remove 125I-Aβ that remained on cell surface. The remaining radioactivities in TR-BBB cells that represent 125I-Aβ were calculated as cell/medium ratio (μg/mg) (see “Experimental Procedures”). Incubation with 125I-Aβ at 37 °C elicited a rapid and robust uptake of 125I-Aβ by TR-BBB cells with total uptake reaching a plateau within 8 min (Fig. 1A). This rapid uptake of 125I-Aβ was not observed at 4 °C, suggesting that the Aβ uptake is a receptor-mediated event. The uptake was competed by the addition of excess unlabeled synthetic Aβ-(1–40) in a concentration-dependent manner, with an IC₅₀ of ~600 nM (Fig. 1B). Because the uptake assays
were performed in serum-free medium, it is unlikely that Aβ uptake requires a prior complex formation of Aβ with serum proteins (e.g. apoE or α2-macroglobulin) (35–38).

To more rigorously estimate the amount of internalized Aβ into TR-BBB cells, we further treated TR-BBB cells with Pronase after incubation with 125I-Aβ at 37 °C to remove surface-bound Aβ. We defined “cell-bound” Aβ as the sum of surface-bound Aβ that includes the binding to specific receptor(s) as well as nonspecific binding and defined “internalized” Aβ as the amount of Aβ taken up into cells and resistant to Pronase treatment (Fig. 1C). TR-BBB cells retained certain levels of 125I-Aβ radioactivity even after Pronase treatment, which was reduced by co-incubation with unlabeled Aβ-(1–40), confirming that Aβ is actually internalized into cells (Fig. 1D). We also found that unlabeled Aβ-(1–42) blocked internalization of 125I-Aβ-(1–40) with an efficacy similar to that of Aβ-(1–40) (Fig. 1D). Taken together, our data show that TR-BBB cells rapidly internalize Aβ possibly through a receptor-mediated mechanism and that Aβ-(1–40) and Aβ-(1–42) probably share the same receptor.

**TR-BBB Cells Partially Degrade Internalized Aβ and Release Fragments and Intact Aβ into Culture Medium**—To examine the fate of internalized Aβ, we performed an efflux assay. TR-BBB cells were first incubated with 125I-Aβ for 10 min, and the media were then replaced with the ECF buffer. Cells were further incubated at 37 °C for various periods of time to allow efflux of Aβ. We found that the cell/medium ratio of Aβ internalized into TR-BBB cells was rapidly decreased over the incubation time (shown as cell in Fig. 2A). Concurrently, the radioactivity in the incubation medium was increased (shown as medium in Fig. 2A), suggesting that the internalized Aβ is rapidly effluxed into the culture medium (Fig. 2A). We next precipitated Aβ released into culture medium by trichloroacetic acid and found that ~80% of 125I-Aβ in the culture medium escaped trichloroacetic acid precipitation and that the ratio of 125I-Aβ precipitated by trichloroacetic acid was constant for 15 min (Fig. 2B). However, SDS-PAGE analysis of culture medium revealed that a portion of effluxed Aβ remained intact and migrated at 4 kDa, which increased in a time-dependent manner (Fig. 2C). These data suggest that a significant proportion of internalized Aβ was degraded within TR-BBB cells before being released into the extracellular space.

**Aβ Internalization Is Inhibited by RAP or Anti-LRP1 Antibody**—We next examined whether LRPI plays a major role in the internalization of 125I-Aβ into TR-BBB cells. We conducted the experiments in the presence of RAP, an antagonist of low density lipoprotein receptor family proteins, including LRPI (39). When TR-BBB cells were incubated with 125I-Aβ in the presence of RAP, the uptake of 125I-Aβ was significantly inhibited (Fig. 3A). The strong inhibitory effect of Aβ uptake by RAP was observed even after Pronase treatment. The internalization of 125I-Aβ by TR-BBB cells was significantly inhibited by RAP, at an extent of ~90% of that inhibited by unlabeled Aβ-(1–40) (Fig. 3B). Aβ binding to the cell surface was also blocked by RAP, at an extent of ~95% of that by unlabeled Aβ-(1–40), supporting the notion that the major proportion of Aβ internalization is a RAP-sensitive process.

It has been suggested that apoE is involved in Aβ clearance (36, 40). To examine the effect of apoE on 125I-Aβ internalization, we prepared an 125I-Aβ-apoE complex by prein-
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FIGURE 3. Inhibition of the Aβ internalization by RAP in TR-BBB cells. A, the uptake assay of [125I]-Aβ or [125I]-AβapoE complex in the absence or presence of 500 nM RAP. The mean ± S.E. in four independent assays is shown. **, p < 0.01; ANOVA. B, the internalized and cell-bound [125I]-Aβ in TR-BBB cells were measured in the absence or presence of 500 nM RAP and 3 μM unlabeled Aβ(1–40). The mean ± S.E. in six independent assays is shown. **, p < 0.01; ANOVA. C, the uptake assay of [125I]-Aβ was performed with 0, 10, 30, 62.5, 125, 300, 500, and 1000 nM RAP in the uptake medium. The mean ± S.E. in 3–7 independent assays is shown. The uptake assay of [125I]-Aβ in the presence of 3 μM unlabeled Aβ(1–40), 10 μg/ml anti-RAGE antibody, 10 μg/ml control IgG, or 100 μM verapamil. The mean ± S.E. in four independent assays is shown.

FIGURE 4. Anti-LRP1 antibody reduced Aβ internalization in TR-BBB cells. A, the internalized [125I]-Aβ in TR-BBB cells was measured in the presence of anti-LRP1 antibody (RRR) or control IgG (each 160 μg/ml). The mean ± S.E. in 5–6 independent assays is shown. **, p < 0.01; ANOVA. B, the uptake assay of [125I]-Aβ was performed in the presence of 0, 5, 10, 20, 40, 80, 160, and 320 μg/ml RRB in the uptake medium. The mean ± S.E. in three independent assays is shown.

We further examined the possibility of involvement of other Aβ receptor candidates. RAGE and P-gp were reported to mediate the Aβ transport across the BBB from blood to brain (42) and brain to blood (43), respectively. Neither anti-RAGE antibody, which is known to inhibit RAGE function, nor verapamil, a major substrate of P-gp that works as a competitor, affected the internalization of Aβ, excluding an involvement of RAGE and P-gp in the Aβ uptake by TR-BBB cells (Fig. 3D).

We further performed Aβ uptake experiments in the presence of a neutralizing antibody that specifically inhibits LRP1 function. Western blot analysis revealed that an antibody RRR, generated against LRP1 holoprotein purified from human placenta, specifically recognized the heavy chain of endogenous LRP1 in TR-BBB cells (data not shown). Coincubation with RRR robustly inhibited Aβ uptake by TR-BBB cells at an extent of ~69% of that inhibited by RAP, in a concentration-dependent manner (Fig. 4, A and B). Aβ binding to the cell surface was also inhibited by RRR at ~32% compared with that in the presence of control immunoglobulin (data not shown). These data strongly support the notion that Aβ internalization into TR-BBB cells is mediated by LRP1.

RNAi Knockdown of Endogenous LRP1 Reduced Aβ Internalization in TR-BBB Cells—To further confirm the involvement of LRP1 in the uptake of Aβ into TR-BBB cells, we knocked down LRP1 in TR-BBB cells by siRNA treatment using two stealth RNAis, a chemically modified RNA molecule that eliminates the induction of interferon pathway, against LRP1 and a stealth negative control RNA. TR-BBB cells were incubated with siRNAs against LRP1 for 30 h. The expression levels of LRP1 in TR-BBB cells were significantly reduced by the two LRP1-specific siRNAs to ~34% and ~29% of the levels by a control siRNA (Fig. 5A). We incubated the siRNA-treated and control cells with [125I]-Aβ and found that the internalization of Aβ by TR-BBB cells was reduced in siRNA-treated cells by ~38% (siRNA 1) and ~52% (siRNA 2) of that inhibited by RAP (Fig. 5B). Cell-bound Aβ was also reduced by siRNA treatment for LRP1 (Fig. 5C). These data strongly suggest that LRP1 is involved in the uptake of Aβ by TR-BBB cells. We also confirmed that siRNAs against LRP1 inhibited internalization of [125I]-tPA, one of the well known ligands of LRP1, in TR-BBB cells (Fig. 5D).

Lack of Rapid Uptake of Aβ by MEF Cells That Express Endogenous LRP1—To further examine the role of LRP1 in the cellular internalization of Aβ, we performed Aβ uptake assays in MEFs derived from LRP1-deficient and wild-type mice (32). An anti-LRP1 antibody, R488, revealed the expression of light chain of LRP1 in MEF-1 cells derived from wild-type mice, whereas PEA10 cells derived from LRP1 heterodeficient mice exhibited ~50% levels of LRP1 compared with that of wild-type mice, and PEA13 cells derived from LRP1 homodeficient mice completely lacked the expression of LRP1 (Fig. 6A). Unexpectedly, however, neither the MEF-1 (wild-type), PEA10, nor PEA13 cells exhibited any significant uptake of Aβ that was observed in TR-BBB cells (Fig. 6B). Furthermore, we confirmed that the levels of cell surface binding of Aβ were at similar levels in either of the MEF cell lines (data not shown), suggesting that MEF cells fail to bind and internalize Aβ regardless of the expression levels of LRP1.

To see whether this inability of Aβ internalization was due to the low expression levels of functional LRP1 in these MEF cells, we performed a tPA internalization assay. As shown in Fig. 6C, three MEF cell lines took up [125I]-tPA at levels proportional to
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Aβ Does Not Directly Bind LRP1 in Vitro—Based on these data, we reasoned that the expression of functional LRP1 per se is not sufficient for the binding and internalization of Aβ. We thus reexamined the binding of Aβ to LRP1 in an in vitro assay. LRP1 holoprotein purified from human placenta was immobilized to microtiter plates, and the binding of Aβ to LRP1 was evaluated by an overlay assay. tPA and apoE, both being authentic ligands of LRP1, exhibited robust binding to immobilized LRP1 holoprotein, which was significantly blocked by RAP. Unexpectedly, however, Aβ did not show any significant binding to LRP1 either in the presence or in the absence of RAP (Fig. 7A).

We also performed similar sets of binding experiments using recombinant fragments of ligand-binding clusters of LRP1 (i.e. sLRP2 and sLRP4) (Fig. 7B). tPA bound to immobilized sLRP2 and sLRP4 in a dose-dependent manner (Fig. 7C), whereas no binding of Aβ to sLRP2 and sLRP4 was detected (Fig. 7D). Altogether, these data argue against the notion that Aβ directly interacts with LRP1.

**DISCUSSION**

To gain insights into the molecular and cellular mechanism of Aβ transport across the BBB, we established a novel cellular model using an immortalized brain endothelial cell line, TR-BBB cells, that recapitulates the robust and rapid uptake of Aβ and found the following: 1) TR-BBB cells rapidly internalize chaperone-free Aβ by a receptor-mediated mechanism; 2) the internalized Aβ is rapidly degraded and released into the medium; 3) the internalization of Aβ into TR-BBB cells is mediated by LRP1, although Aβ may not directly bind LRP1 on the cell-surface. Based on these findings, we hypothesize that BMECs that delineate BBB rapidly internalize chaperone-free Aβ peptides in the brain parenchyma and efflux them into blood by LRP1-dependent transcytosis.

Internalization of soluble or fibrillar forms of Aβ has been documented in several types of cells (13, 35–38, 40, 45, 46, 48). However, the most striking difference between the previous reports and the present observation consists in the time course of Aβ uptake; Aβ internalization took place over a long incuba-
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FIGURE 7. Lack of direct binding of Aβ to LRP1 in vitro. A, the binding of full-length human LRP1 purified from placenta and coated onto microtiter plates to human Aβ(1–40) and tPA in the absence or presence of 1 μM RAP, respectively. The mean ± S.E. of optical densities (450 nm) in four independent assays is shown, **, p < 0.01. B, the CBB staining of purified recombinant sLRP2 and sLRP4. C, the binding of increasing concentrations of tPA to immobilized sLRP2, sLRP4, or bovine serum albumin. The mean ± S.E. of optical densities (450 nm) in four independent assays is shown, **, p < 0.01. D, the binding of increasing concentrations of Aβ to immobilized sLRP2, sLRP4, or bovine serum albumin. The mean ± S.E. of optical densities (450 nm) in four independent assays is shown. N.S., not significant; BSA, bovine serum albumin.

In contrast, the involvement of other known Aβ transporters (e.g. low density lipoprotein receptor, which is known as the major receptor for apoE) were involved; RAP might have failed to efficiently block the uptake of Aβ-lipidated apoE complex because of the relatively low affinity of other apoE receptors for RAP compared with that of LRP1.

Despite the requirement of LRP1 in Aβ uptake by TR-BBB cells, Aβ uptake was not observed in the MEF cells, although the expression of functional LRP1 that can mediate tPA internalization was demonstrated (Fig. 6B). We also tested Aβ uptake in a range of cell lines but failed to observe the rapid LRP1-dependent Aβ internalization in any cells other than TR-BBB (supplemental Fig. 2). These data suggest that LRP1 is required, but not sufficient, to cause a rapid internalization of Aβ and that cell type specificity may significantly affect the LRP1-dependent Aβ internalization. Because we could not detect the direct binding of Aβ to LRP1 in a series of carefully controlled in vitro binding assays using full-length LRP1 or ligand-binding cluster fragments thereof (Fig. 7), we speculate that this specificity of LRP1-dependent internalization of Aβ may best be explained by the intercalation of an Aβ-binding molecule (including cell surface LRP1 ligands) that is specifically expressed on the surface of TR-BBB cells and cooperates with LRP1 to internalize Aβ into cells.

LRP1 is known to function as a multifunctional receptor that recognizes at least 30 different ligands, although the mechanism regarding how LRP1 can recognize structurally unrelated ligands remains unknown (51, 52). It is noteworthy that LRP1 directly binds to a few of these molecules and that many of the ligands interact with their “co-receptor” and are subsequently internalized via LRP1. For example, urokinase-type plasminogen activator and PAI-1 initially bind urokinase-type plasminogen activator receptor, and then the heterotrimeric complex is rapidly internalized by LRP1 and catabolized (53). Also, factor VIII initially adheres to heparan sulfate proteoglycans on the surface of the hepatocytes, and the complex is then scavenged by LRP1 (54). Based on these previous observations, it is plausible that an as yet unidentified Aβ-binding molecule that acts as a co-receptor of LRP1 may best explain the unique feature of TR-BBB cells, which robustly internalize chaperone-free Aβ (supplemental Fig. 3). Among the known LRP1 co-receptors, heparan
sulfate proteoglycans have been shown to bind Aβ (55), although treatment of TR-BBB cells with heparin or heparanase or conrodisin did not affect the Aβ uptake, ruling out the possibility that heparan sulfate proteoglycans or chondroitin sulfate proteoglycans represent the cell surface co-receptor for Aβ in TR-BBB cells (supplemental Fig. 4). Notably, HUVEC cells derived from umbilical veins failed to show Aβ uptake, and the expression level of LRP1 in HUVEC cells was extremely low (supplemental Fig. 2). This may support the crucial role of LRP1 in the rapid Aβ internalization in brain endothelial cells. However, it is still possible that some cellular environments, other than an Aβ binding co-receptor, are the major determinants for Aβ internalization in TR-BBB cells. The nature of the cell type specificity for Aβ uptake should further be addressed.

In contrast to our results, previous studies showed that recombinant LRP1 polypeptides, especially the cluster II and IV domains, directly bind Aβ in vitro by the surface plasmon resonance analysis (18, 47). Although the reason for this discrepancy is not clear, we believe that our present data based on the plate binding assays that have been extensively used for the characterization of ligand-LRP1 (27) as well as ligand-Aβ (44) interactions, using purified LRP1 and appropriate controls, are quite reliable so far as in vitro interactions are concerned.

Because of the lack of polarity as well as formation of tight junctions in TR-BBB cells, we cannot fully replicate the “trans-transport” across the BBB; in this regard, what we have analyzed here may be crucial to the understanding of the pre-transport across the BBB as well as small molecule compounds that facilitate the TR-BBB cells may well recapitulate the initial internalization step of the Aβ transport in vivo.

Another problem regarding the role of LRP1 in Aβ clearance is whether LRP1 mediates transcytosis or degradation of Aβ, as Nazer et al. (46) studied in polarized Madin-Darby canine kidney cells. Indeed, our trichloroacetic acid precipitation assay suggested that ~80% of Aβ released from TR-BBB cells underwent degradation in the Madin-Darby canine kidney cells (46). However, further study is required for the characterization of transcytosis versus degradation of Aβ in relation to the time course and cell type specificity.

In summary, we established a new in vitro model of rapid Aβ internalization using TR-BBB cells. We believe that Aβ internalization by TR-BBB may recapitulate some aspects of the initial internalization step of Aβ transport across the BBB. Stimulation of the Aβ efflux from brain to blood may be a useful strategy to inhibit Aβ accumulation and prevent the development of pathological changes in AD. TR-BBB cells may serve as a useful tool for the screening of modulation of proteins or genes participating in Aβ transport at the BBB as well as small molecule compounds that facilitate the Aβ clearance. The identification of the mechanism of the cell type specificity in Aβ internalization may be crucial to the understanding of the precise mechanism of Aβ transport across the BBB.

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LRP1-mediated Aβ Clearance in TR-BBB Cells


The Low Density Lipoprotein Receptor-related Protein 1 Mediates Uptake of Amyloid β Peptides in an in Vitro Model of the Blood-Brain Barrier Cells

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