Angiotensin-converting Enzyme Degrades Alzheimer Amyloid β-Peptide (Aβ); Retards Aβ Aggregation, Deposition, Fibril Formation; and Inhibits Cytotoxicity

We have demonstrated that the angiotensin-converting enzyme (ACE) genotype is associated with Alzheimer’s disease (AD) in the Japanese population (1). To determine why ACE affects susceptibility to AD, we examined the effect of purified ACE on aggregation of the amyloid β-peptide (Aβ) in vitro. Surprisingly, ACE was found to significantly inhibit Aβ aggregation in a dose response manner. The inhibition of aggregation was specifically blocked by preincubation of ACE with an ACE inhibitor, lisinopril. ACE was confirmed to retard Aβ fibril formation with electron microscopy. ACE inhibited Aβ deposits on a synthaloid plate, which was used to monitor Aβ deposition on autopsied brain tissue. ACE also significantly inhibited Aβ cytotoxicity on PC12 h. The most striking fact was that ACE degraded Aβ by cleaving Aβ-(1–40) at the site Asp7-Ser8. This was proven with reverse-phase HPLC, amino acid sequence analysis, and MALDI-TOF/MS. Compared with Aβ-(1–40), aggregation and cytotoxic effects of the degradation products Aβ-(1–7) and Aβ-(8–40) peptides were reduced or virtually absent. These findings led to the hypothesis that ACE may affect susceptibility to AD by degrading Aβ and preventing the accumulation of amyloid plaques in vivo.

Progressive cerebral dysfunction in Alzheimer’s disease (AD) is accompanied by innumerable extracellular amyloid deposits in the form of senile plaque and microvascular amyloid. Amyloid protein is derived from the integral membrane polypeptide, β-amyloid precursor protein (βAPP). The released 39–43 residue amyloid β-peptide (Aβ) may subsequently undergo aggregation to form amyloid fibrils under the influence of various amyloid-associated factors (2). The aggregation and deposition of Aβ has been linked to the toxic effects causing cell damage in AD. Because Aβ is present in both normal and AD subjects, an answer to the question of why Aβ accumulates in AD but not in the normal brain may lead to a possible cure for AD.

Angiotensin-converting enzyme (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1) is a membrane-bound ectoenzyme. It catalyzes the conversion of angiotensin I (AngI) to angiotensin II (AngII), which plays an important role in blood pressure and body fluid and sodium homeostasis (3). The cloning of the ACE gene revealed a 287-bp insertion (I/deletion (D) polymorphism in intron 16. The serum ACE activity of the ACE DD genotype was twice as high as that of the ACE II genotype (4). The ACE genotype is considered to be associated with hypertension, coronary artery disease, left ventricular hypertrophy, myocardial infarction, and diabetic nephropathy (5–7). In particular, the ACE DD genotype is considered to be a risk factor for vascular diseases.

We have compared the distribution of an I/D polymorphism of the gene coding for ACE in 133 Japanese sporadic AD patients and 257 control subjects (1). The association between AD and ACE genotypes or alleles was found to be significant. The frequency of the ACE II genotype was 1.4 higher in AD than in controls, whereas that of ACE DD genotypes was only 0.4× as high. Moreover, the altered distribution of ACE alleles with AD patients appears to be independent of ApoE (1). The association between AD and ACE genotypes was even more significant in the Japanese population than in the British population (8). Although several reports published recently elucidate the association between ACE genotype and AD (9–11), the mechanism of how ACE influences susceptibility to AD remains unclear. Here, we provide the first evidence that ACE significantly inhibits the aggregation, deposition, and cytotoxicity of Aβ in vitro by degrading Aβ-(1–40) at the site Asp7-Ser8.

EXPERIMENTAL PROCEDURES

Preparation of ACE and Immunoblotting—Somatic ACE was purified from human seminal plasma by using lisinopril-coupled Sepharose as described (12). Immunoblotting was done with anti-somatic ACE antibodies as described (12).

ACE Activity Assay—Enzymatic activity of ACE was determined with the ACE color kit (Fujirebio, Japan) in which p-hydroxyhippuryl-L-histidy-L-leucine was used as substrate (13). ACE activity was monitored by absorbance at 505 nm.

Inhibition of ACE Activity by Lisinopril—Lisinopril was added to fixed amounts of PBS-diluted seminal plasma to the final concentrations described in the legend to Fig. 2. After incubation for 15 min at room temperature, ACE activities were determined.

Aggregation Studies—Synthetic Aβ (1–40, 1–7, 8–40) (Peptide Institute, Osaka, Japan) was dissolved first in dimethyl sulfoxide (MeSO) and then in PBS to form the stock solution (1 mM Aβ containing 25% MeSO). The stock solution was diluted 10-fold with PBS and incubated with or without ACE at 37 °C for 4 days. Aggregation of Aβ was measured by adding 10 μl of Aβ solution into 0.5 ml of thioflavin T (TTh) solution (final concentration: 3 μM in 50 mM sodium phosphate buffer, pH 6.0) and measuring the fluorescence intensity (λex at 450 nm, λem at 482 nm).

Aβ Deposition Assay—Various concentrations of ACE were incubated with 10 nCi of 125I-Aβ (Amersham Biosciences) in 100 μl of TE buffer (50 mM Tris, pH 7.5, containing 0.1% BSA) at 37 °C for 3 h. Then the...
resulting solution was incubated in a Synthaloid Drug Screening Plate (Quality Controlled Biochemicals Inc.). The deposited Aβ was detected as radioactive signals according to the manufacturer’s instructions.

Electron Microscopy—100 μM Aβ solutions containing 2.5% MeSO and preincubated with or without ACE and lisinopril (as prepared in the aggregation studies) were examined. The fibril-formed peptide in the solutions was adsorbed onto 200-mesh Formvar-coated copper grids and negative-stained with 2% uranyl acetate. The fibrils were observed with an electron microscope at 80 kV.

Cytotoxicity Assay—Rat pheochromocytoma PC12 h cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum, 10% fetal calf serum, 2 mM l-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C under 5% CO₂. For the neurotoxicity assay, cultured PC12 h cells were seeded onto a 96-well plate at a density of 10⁴ cells/100 μl/well in a serum-free medium supplemented with 2 μM insulin. The cell counting kit-8 (Dojindo, Kumamoto, Japan) was used to measure the activities of dehydrogenase enzymes in living cells according to the manufacturer’s instructions. Briefly, 10 μl of synthetic Aβ, preincubated with or without ACE, were added to each well. After incubation for 3 days, 10 μl of 5 mM WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) containing 0.2 mM 1-methoxy-5-methyl-phenazinium-methyl-sulfate, and 150 mM NaCl was added to each well, followed by another hour of incubation. The WST-8 reduction was determined colorimetrically at 450 nm using an automatic microplate spectrophotometer.

Reverse Phase HPLC—Fifty microliters of the reaction mixture was injected onto a TSK gel ODS120T column (0.64 × 25 cm, particle size 5 μm) and eluted at 1 ml/min with a linear gradient of 0–80% acetonitrile, over a period of 50 min. The peaks monitored at 210 nm were collected.

Amino Acid Sequence—Microsequencing was performed automatically by a gas-liquid sequencer (Shimadzu, model PSQ1). Phenylthiohydantoin (PTH)-derivatives were identified by Shimadzu LC system and negative-stained with 2% uranyl acetate. The fibrils were observed with an electron microscope at 80 kV.

Fig. 1. Inhibition of ACE activity by lisinopril. A serially diluted solution of lisinopril was added to ACE prepared from seminal plasma. After incubation, ACE activities were measured.

Fig. 2. Inhibition of Aβ aggregation by ACE. One hundred microliters of Aβ at a final concentration of 100 μM was incubated for 4 days at 37 °C with 240 milliunits ACE, 160 milliunits ACE, 60 milliunits ACE, 240 milliunits ACE pretreated with 10 μM lisinopril for 15 min, 250 μg/ml of BSA or PBS. Aβ aggregation was measured with the ThT binding method. The values are the means ± S.D. (n = 3). *, p < 0.05; ***, p < 0.001.

RESULTS
Preparation of ACE—Somatic ACE is present in serum and seminal plasma. We measured the ACE activity in human seminal plasma (844.84 ± 344.27 units/liter (n = 139)). In contrast, normal human serum ACE activity has been reported to be 7.60 ± 2.01 units/liter (n = 173) (14). Because the activity in seminal plasma is over 100-fold higher than that in serum, we purified ACE from seminal plasma using the ACE inhibitor, lisinopril, as an affinity ligand. The purity of ACE eluted from the lisinopril-coupled Sepharose column was confirmed by electrophoregram. Purified ACE showed a single band with a molecular mass of 180 kDa using Coomassie Blue staining, and this band was strongly recognized by the anti-somatic ACE monoclonal antibody in immunoblotting (Fig. 1). The purified ACE had an activity of about 20 unit/mg of protein that could be inhibited by lisinopril at a final concentration ranging from 10 to 0.01 μM in a dose response manner (Fig. 2).

ACE Inhibited Aβ Aggregation—Synthetic Aβ in aqueous buffer tends to self-aggregate (15, 16), and only self-aggregated Aβ exerts cytotoxicity. We detected Aβ aggregation quantitatively using fluorescence of ThT, a reagent that associates rapidly with aggregated Aβ but not with monomeric or dimeric Aβ, giving rise to a new excitation absorption at 450 nm (17). As shown in Fig. 3, 100 μM Aβ solution aggregated remarkably after incubating at 37 °C for 4 days. When Aβ solution was incubated with ACE, the aggregation was significantly inhibited, and the inhibition was dose-dependent. A concentration of 240 milliunits/100 μl ACE reduced Aβ aggregation to about 20% of the control (p = 1.7 × 10⁻⁵ versus PBS). The presence of 2.5% MeSO in the solution did not affect Aβ aggregation (data not shown).

To elucidate whether the inhibition was specific, a final concentration of 10 μM of lisinopril, which could inhibit about 98% of ACE activity (Fig. 2), was added to the ACE solution 15 min before incubation with Aβ. As shown in Fig. 3, pretreat-
Affect cell survival (50 °C for 3 h). After incubation for 3 days, the values are the means ± S.D. (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001.

57 ± 2% (p = 0.005 versus ACE (240 milliunits)). These data suggest that the inhibitory effect on Aβ cytotoxicity was a specific effect by ACE.

Degradation of Aβ-(1–40) Occurred During Incubation with ACE—To investigate the reason ACE affected Aβ aggregation
and cytotoxicity, we tried to determine if any degradation occurred during the incubation of Aβ-(1–40) with ACE and discovered a new degraded fragment using an HPLC chromatogram (Fig. 7). The degraded fragment was eluted at a more hydrophobic region compared with Aβ-(1–40) (Fig. 7, A and B). Amino acid sequence analysis showed that the first ten residues of the degraded fragment (Fig. 7A, peak b) was SGYEVHQKL, which corresponded to Aβ-(8–17). The elution time of the degraded fragment coincided with that of synthetic Aβ-(8–40) peptide (peak in C) and degraded Aβ-(1–7) (peaks in E). The elution time of peak a in A coincided with that of synthetic Aβ-(1–40) peptide (peak in B).

To confirm that the degraded fragment is Aβ-(8–40), we incubated Aβ-(1–7) with ACE. The HPLC plot revealed that Aβ-(1–7) was further degraded. The Aβ-(1–7) peak (Fig. 7D) disappeared after incubation with ACE and was replaced by three small peaks (Fig. 7E). The three small peaks could also be detected after incubating Aβ-(1–40) with ACE (Fig. 7A).

Degradation Products of Aβ Showed Less Aggregation and Low Cytotoxicity—Aggregation and cytotoxic effects of the degradation products were investigated simultaneously with Aβ-(1–40). Aβ-(1–7) showed neither an aggregation nor a cytotoxic effect. Aβ-(8–40) gave an aggregation effect of 35.7%, which was significantly lower than that of Aβ-(1–40). The level of aggregation of Aβ-(8–40) incubated with Aβ-(1–7) was not significantly different from that of Aβ-(8–40) alone (Fig. 8). As shown in Fig. 9, PC12 h cells incubated with Aβ-(8–40) for 3 days exhibited a reduced survival to 77 ± 3%, which was significantly higher than that seen with Aβ-(1–40) (p = 0.004 versus PBS).
The recent studies on the renin-angiotensin system (RAS) of the mammalian brain may explain the association between ACE and AD in a certain sense. Besides the classical RAS, a local RAS in the brain may play a critical role in the central nervous system. It has been reported that angiotensin in astrocytes is required for the functional maintenance of the blood brain barrier (23), which is impaired in AD (24). Central RAS prevents neuronal cells from apoptosis not only by AngII but also by AngIV, an AngII metabolite (25). Both AngII and AngIV excite hippocampal neuronal activity (26) and regulate cerebral blood flow (27). Colocalization of ACE and Ang1 receptor in the substantia nigra, the caudate nucleus, and putamen of human and rat suggests central RAS may be important in modulating central dopamine release. In Parkinson’s disease, there is a marked reduction of ACE receptors associated with the nigrostriatal dopaminergic neuron loss, and ACE inhibitor modifies the clinical features of Parkinson’s disease (28). The striking distribution of AngIV receptors in cholinergic neurons, motor, and sensory nuclei of the brain suggest that AngIV plays an important role in the facilitation of learning and memory (29–31). These studies demonstrate that angiotensin is essential not only to the circulatory system, but also to the central nervous system. Although these results are helpful in understanding the relationship between AD and ACE, they do not provide direct evidence.

AD is a heterogeneous disorder with a variety of molecular pathologies converging predominantly on abnormal amyloid deposition particularly in the brain. Aβ aggregation into senile plaques is an important pathological hallmark of AD. We hypothesize that ACE may affect Aβ aggregation and deposition in the brain. We have substantiated the hypothesis and elucidate here that ACE inhibits Aβ aggregation, deposition, fibril formation, and cytotoxicity in vitro. These results provide the first evidence of direct involvement of ACE with AD susceptibility.

Several lines of evidence have shown that amorphous, largely nonfilamentous deposits of Aβ (so called “diffuse” or preamyloid plaques) precede the development of fibrillar amyloid, dystrophic neurites, neurofibrillary tangles, and other cytopathological changes in Down’s syndrome and AD. In the AD brain, diffuse plaques composed mostly of amorphous Aβ are inert, whereas compact plaques composed of Aβ fibrils are associated with neurodegenerative changes (32, 33). In vitro experiments also reveal that the neurotoxicity of Aβ is associated with their ability to form stable aggregates in aqueous solution (34–36). The aggregation of Aβ only is not sufficient to exert neurotoxicity effect, but further amyloid fibril formation is required (37). Aggregation of Aβ is template-independent initial nidus formation, and deposition of Aβ is template-dependent subsequent to plaque growth. These are considered fundamentally distinct biochemical processes in AD (38). Taken together, these findings suggest that aggregation, deposition, and fibril formation are the necessary processes for Aβ to achieve and strengthen a neurotoxic state. Just in these critical processes, ACE plays an important role in decrease of
Aβ neurotoxicity, suggesting the possible cause of ACE genotype in affecting susceptibility to AD.

Three types of proteases, which are designated α-, β-, and γ-secretases, cleave APP. Processing by α-secretase cleaves within the Aβ sequence whereas β- and γ-secretase cleaves on the N- and C-terminal ends of the Aβ region, respectively, releasing Aβ (39). γ-Secretase cleaves at several adjacent sites to yield Aβ species containing 39–43 amino acid residues. Because α-secretase destroys the Aβ sequence, it is generally thought that α-secretase pathway mitigates amyloid formation, although this has not yet been demonstrated unequivocally (40). In addition, the C-terminally truncated form of APP released by α-secretase may have trophic actions (41), which could antagonize the neurotoxic effects of aggregated Aβ (42). ACE acts like the α-secretase in degrading Aβ and thus preventing aggregation.

Several Aβ-degrading enzymes were studied because of their potential usage in AD treatment. BACE can cleave full-length APP at Asp3 of the Aβ sequence and also at Glu11 (42). Recently, Cheseune et al. (43) reported that insulin-degrading enzyme (IDE) is sufficient to degrade Aβ (43). Ivata et al. (44) reported that endopeptidase 24.11 (neprilisin) is involved selectively in the catabolism of Aβ (1–42) in rat brain parenchyma. Yu et al. (45) reported that mid-kine formed complexes with Aβ (1–40) and protected PC12 h from Aβ-induced cytotoxicity.

In the present study, we report that ACE is a new Aβ-degrading enzyme that cleaves the Aβ sequence at Asp3-Ser9. The cleavage site is different from the site that converts AngI to AngII, or other Aβ-cleaving sites reported as yet. Although the actual meaning of a real Aβ-degrading function of ACE in vivo remains to be further studied, our data strongly lead to the hypothesis that ACE may affect susceptibility to AD by degrading Aβ and preventing the accumulation of amyloid plaques in the brains of AD patients.

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