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$A\beta_{43}$ Is the Earliest-Depositing $A\beta$ Species in APP Transgenic Mouse Brain and Is Converted to $A\beta_{41}$ by Two Active Domains of ACE

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Amyloid- β protein ($A\beta$) varies in length at its carboxyl terminus. The longer $A\beta$ species, $A\beta_{43}$ and $A\beta_{42}$, are highly amyloidogenic and deposit more frequently than $A\beta_{40}$ in the brain of Alzheimer disease (AD) patients. However, the characterization of $A\beta_{43}$ deposition in the brain and the relationship between $A\beta_{43}$ and $A\beta_{42}$ or $A\beta_{40}$ remain unclear. We provide evidence that $A\beta_{43}$ deposition appears earlier than $A\beta_{42}$ and $A\beta_{40}$ deposition in the brain of mutant amyloid precursor protein transgenic (APPtg) mice, suggesting that $A\beta_{43}$ is the earliest-depositing species. In addition, we found increased $A\beta_{43}$ levels and $A\beta_{43}/A\beta_{42}$ ratios in the serum of AD patients, suggesting their use as diagnostic blood biomarkers for AD. We further show that angiotensin-converting enzyme (ACE) converts $A\beta_{43}$ to $A\beta_{41}$. Notably, this $A\beta_{43}$ -to- $A\beta_{41}$ converting activity requires two active domains of ACE. Inhibition of ACE activity significantly enhanced $A\beta_{43}$ deposition in APPtg mouse brain. Our results suggest that $A\beta_{43}$ is the earliest-depositing species in brain parenchyma and that $A\beta_{43}$ may trigger later $A\beta_{42}$ and $A\beta_{40}$ deposition or may be converted to $A\beta_{42}$ and $A\beta_{40}$ plaques. Activities of both ACE domains may be important for reducing $A\beta_{43}$ levels in serum and reducing brain $A\beta_{43}$ deposition. (*Am J Pathol* 2013, 182: 2322–2331; <http://dx.doi.org/10.1016/j.ajpath.2013.01.053>)

Alzheimer disease (AD) is the most common form of dementia in the elderly, accounting for 50% to 60% of all cases. Amyloid plaques and neurofibrillary tangles are two major characteristic pathological features of AD. The major components of extracellular amyloid plaques are amyloid- β proteins ($A\beta$) consisting of 40 to 42 or even 43 amino acids, which are generated from amyloid precursor protein (APP) by β -secretase and γ -secretase.¹ $A\beta_{40}$ is the major species, comprising 90% of all secreted $A\beta$, with $A\beta_{42}$ comprising 5% to 10%. Until recently, all $A\beta$ species have been considered pathogenic molecules of AD; however, there is evidence to suggest that $A\beta_{40}$ is not the pathogenic form and that it even inhibits the neurotoxicity and plaque formation of $A\beta_{42}$.² Nonetheless, intensive research has demonstrated that the major $A\beta$ species deposited in the plaques is $A\beta_{42}$ and that the progressive accumulation of neurotoxic $A\beta_{42}$ plays the central role in the genesis of AD.³ Presenilins 1

and 2 (PS-1 and PS-2) are the catalytic components of the γ -secretase complex. Missense mutations associated with early-onset familial AD are found in the *APP*, *PSEN1*, and *PSEN2* genes, and these mutations lead variously to increased $A\beta_{42}$ production or decreased $A\beta_{40}$ production.⁴

In addition to $A\beta_{42}$, there is another longer species: $A\beta_{43}$. Recent studies reveal that $A\beta_{43}$ deposits more frequently than $A\beta_{40}$ not only in familial AD brain, but also in sporadic AD brain and Down's syndrome brain.^{5,6} $A\beta_{43}$ has stronger neurotoxicity than $A\beta_{42}$ and is overproduced by expression

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of familial AD—associated *PSEN1* mutations in cells.⁷ These lines of evidence suggest that A β ₄₃ also plays an important role in the pathogenesis of AD, in addition to A β ₄₂. However, which is the first-depositing A β species in the brain of APP transgenic mice has been unknown. Here, we report data suggesting that A β ₄₃ deposits in the brain earlier than A β ₄₂ and A β ₄₀. We found increases in A β ₄₃ levels and A β ₄₃/A β ₄₂ ratio in the serum of AD patients, but a tendency toward decreased A β ₄₃ levels and reduced A β ₄₃/A β ₄₀ ratio in the cerebrospinal fluid (CSF) of AD patients. Changes in the A β ₄₃ levels and ratios may serve as diagnostic indexes for AD. Because angiotensin-converting enzyme (ACE) converts A β ₄₂ to A β ₄₀ and because lower ACE activity is associated with the onset of AD,^{8–10} we examined whether ACE can convert A β ₄₃ to a shorter species and whether ACE is involved in lowering A β ₄₃ deposition. Notably, ACE converted A β ₄₃ to A β ₄₁, and this activity requires two active domains of ACE. Furthermore, long-term inhibition of ACE activity with captopril significantly enhanced brain A β ₄₃ deposition, suggesting that ACE activity may help prevent A β ₄₃ deposition in the brain.

Materials and Methods

Animals and Drug Treatment

hAPPSw transgenic Tg2576 mice were purchased from Taconic Farms (Germantown, NY), and platelet-derived growth factor (PDGF)-APPSwInd transgenic J20 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Tg2576 mice were 13 to 21 months of age and J20 mice were 8 to 20 months of age before analysis. Tg2576 mice were housed singly in individual cages and fed with captopril-supplemented diet (0.25 mg/g) or control diet from age 6 months to age 17 months. The average captopril intake per animal was 30 mg/kg body weight per day.⁸ All animal procedures were approved by the Iwate Medical University Committee for Animal Use.

Tissue Preparation and IHC

Mice were euthanized by inhalation of CO₂ and then were transcardially perfused with cold PBS. Postfixed brains were sagittally cut into sections (30 μ m thick) using a cryomicrotome. Brain sections were immunostained with anti-A β ₄₀ monoclonal, anti-A β ₄₂ monoclonal, anti-A β ₄₂ polyclonal, and anti-A β ₄₃ polyclonal end-specific antibodies after a 98% formic acid pretreatment for 3 minutes.⁸ Anti-A β ₄₀ monoclonal, anti-A β ₄₂ polyclonal, and anti-A β ₄₃ polyclonal antibodies were from purchased from IBL Japan (Takasaki, Japan). Anti-A β ₄₂ monoclonal antibody was purchased from Wako Pure Chemical Industries (Osaka, Japan). For immunohistochemistry, brain sections were stained by standard immunoperoxidase techniques with ABC kits (Vector Laboratories, Burlingame, CA) or by immunofluorescence-labeling

procedures with Alexa Fluor 488— and Alexa Fluor 568—tagged secondary antibodies (Life Technologies—Invitrogen, Carlsbad, CA). Two to three sections were counted for each mouse. Images of the brain sections were captured under light microscopy or fluorescence microscopy (Olympus IX81) or under laser scanning confocal microscopy (Olympus FV1000) (Olympus, Tokyo, Japan).

A β ELISA

Human serum and CSF from living AD patients and normal subjects were collected using standard methods. For serum collection, 26 AD patients and 18 age-matched normal control subjects at the Iwate Medical University Hospital were enrolled. The average age of the AD group was 75.1 $\pm$ 1.8 years, and that of the control group was 75.4 $\pm$ 1.1. Clinical diagnosis of AD was based on the NINCDS—ADRDA Alzheimer criteria. The mean Mini-Mental State Examination score of AD patients was 18.6 $\pm$ 1.0. For CSF collection, six AD patients and six age-matched normal controls were enrolled. The samples were aliquoted and stored at -80°C until analyses. A β ₄₂ and A β ₄₀ were measured using enzyme-linked immunosorbent assay (ELISA) kits from Wako Pure Chemical Industries. A β ₄₃ was measured using an A β ₄₃ full-length ELISA kit newly developed by IBL Japan. All samples were measured in duplicate. Experiments using human samples were performed after obtaining the informed consent of the patients or guardians for diagnosis and biochemical, molecular biological, and genomic research. The study was examined and approved by the Ethics Committee of Iwate Medical University Hospital.

Truncated ACE Preparation and ACE Activity Assay

Expression and purification of mutated ACE containing two active domains (F-ACE) or only the N-terminal active domain or C-terminal active domain (N-ACE or C-ACE) was performed as described previously.¹¹ The two zinc metalloprotease active site glutamates (amino acids 362 in the N-domain and 960 in the C-domain) in F-ACE were respectively changed to aspartates to generate F-ACE-E362D and F-ACE-E960D. The following primers were used: F-ACE-E362D, forward 5'-TGCACCATGACATGGCCATATACAG-3' and reverse 5'-ATGGCCCATGT-CATGGTGCACTGTGG-3'; F-ACE-E960D, forward 5'-CACCACGACATGGGCCACATCCAG-3' and reverse 5'-ATGTGGCCCATGTCTGTGGTGGGCCA-3'.

Western Blotting

Synthetic A β _{1–40}, A β _{1–42}, and A β _{1–43} were purchased from Peptide Institute (Osaka, Japan). To examine the specificity of anti-A β ₄₀, anti-A β ₄₂, and anti-A β ₄₃ antibodies, 0.1 μ g of each A β was subjected to tricine-buffered SDS-PAGE using 5% to 20% gradient gel and blotted on a nitrocellulose membrane. To enhance the reactivity to anti-A β antibodies,

the membrane was boiled in PBS for 3 minutes after blotting and then was probed with each A β antibody. Determination of A β_{40} converted from A β_{42} was performed as described previously.¹¹ To examine whether the anti-A β antibodies recognize APP, 40 μ g proteins from brain lysate of adult Tg2576 and J20 mice were subjected to Western blotting using an anti-APP antibody (MAB348; EMD Millipore, Billerica, MA).

Mass Spectrometry Analysis

Mutated ACE proteins were mixed with 80 μ mol/L A β_{43} and incubated at 37°C for 4 hours. Captopril (10 μ mol/L) was added to stop the digestion and the sample was frozen and stored at -80°C until analysis. The samples were mixed with 3,5-dimethoxy-4-hydroxycinnamic acid (Wako Pure Chemical Industries) as a matrix, and then subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (AXIMA-CFR; Shimadzu, Kyoto, Japan) to detect the A β fragments degraded by mutated ACE proteins. The same amount of mutated ACE proteins or A β_{43} incubated alone under the same conditions as described above was used as control.

Statistical Analysis

Statistical analysis was performed using Statview 5.0 software. Data are expressed as means $\pm$ SEM. Statistical significance was set at $P \leq 0.05$. Bonferroni-Dunn test was used to compare means from two or more groups against a control group. Two-tailed Student's *t*-test was used to determine whether the results were significantly different between two groups. Spearman's rank test was used to assess the correlation between brain ACE activity and the number of A β_{43} plaques.

Results

Age-Dependent Deposition of A β_{43} in the Brain of APPtg Mice without PS Mutation

A β_{43} species were found in plaque cores from brain cortex of patients with Down's syndrome, sporadic AD, and familial AD.^{5,6,12} In mouse models, A β_{43} deposition was shown in an APPtg mouse carrying a PS1-R278I mutation that causes overproduction of A β_{43} .⁷ To examine whether A β_{43} deposits in the brain of APPtg mouse without expression of a pathogenic presenilin mutation, we immunostained A β_{43} deposition in brain sections from 13-, 17-, and 21-month-old Tg2576 mice, which carry human Swedish mutant APP. We first examined the specificities of A β antibodies using synthetic A β peptides by Western blotting. A rabbit-derived polyclonal anti-A β_{43} antibody showed high specificity to synthetic A β_{1-43} peptide, and no cross-reactivity to A β_{1-42} and A β_{1-40} was detected (Figure 1A). Similarly, two anti-A β_{42} antibodies derived

Figure 1 A β_{43} deposits in the brain of APPtg mice without presenilin mutation in an age-dependent manner. **A:** Western blot of A β_{40} , A β_{42} , and A β_{43} revealed the high specificity of anti-A β_{40} , anti-A β_{42} , and anti-A β_{43} antibodies. **B–G:** Coronal and sagittal brain sections from 13-, 17-, and 21-month-old Tg2576 mice (**B–D**) were immunostained with anti-A β_{43} antibody. **Boxed regions** in **C** and **D** are shown at higher magnification at the right. Sections from 8-, 14-, and 20-month-old J20 mice were immunostained with anti-A β_{43} (**E–G**) and anti-A β_{42} antibody (**H–J**). The deposition of A β_{43} increased significantly with age and exhibited a pattern similar to that of A β_{42} deposition. **K:** A β_{43} plaques in the hippocampus of 14- and 20-month-old J20 mice were significantly greater in number than in 8-month-old J20 mice. **L:** Western blot of brain lysate from Tg2576 and J20 mice using anti-APP and anti-A β C-terminus antibodies. Data are expressed as means $\pm$ SEM. $n = 6$ or 7. * $P < 0.05$; ** $P < 0.01$ Bonferroni–Dunn test. Scale bar = 500 μ m. HP, hippocampus; PC, piriform cortex; PtAC, parietal association cortex; ST, striatum.

Figure 2 A β_{43} deposition is more abundant and earlier than A β_{40} deposition. **A–Q:** Brain sections from 8-month-old J20 (**A–C**), 20-month-old J20 (**D**), and 21-month-old Tg2576 mice (**E–Q**) were stained for A β_{40} and A β_{43} deposition. Confocal microscopy revealed more extensive distribution of A β_{43} than A β_{40} and a tendency toward expansion of A β_{43} fibrils in diffused plaques (**A–C**). Central localization of A β_{43} deposition in the mature A β plaques is indicated by **arrowheads** (**D, G–I, and L–N**). DIC microscopy revealed that normal brain structure was disrupted by mature plaques (**arrowheads**) with A β_{43} immunoreactivity at the center (**H, I, M, and N**). Central localization of A β_{40} deposition in immature plaques is indicated by **arrowheads** (**O–Q**). **Dashed circles** (**L**) indicate portions in a mature plaque that did not show A β_{40} or A β_{43} immunoreactivity. **R:** Quantitation of A β_{40} and A β_{43} plaque number in the hippocampus and the cortex of Tg2576 mice. Data are expressed as means $\pm$ SEM. $n = 4$. $**P < 0.01$ Bonferroni–Dunn test. Scale bars: 5 μ m (**A–D**); 100 μ m (**E–Q**). cc, corpus callosum (**H**).

from rabbit and mouse and the mouse-derived anti-A β_{40} antibody also showed specificity to A β_{1-42} and A β_{1-40} peptides, respectively (Figure 1A). 6E10 anti-A β antibody recognizing amino acids 1 to 17 of A β showed similar reactivity with A β_{40} , A β_{42} , and A β_{43} (Figure 1A). None of the A β C-terminus–specific antibodies recognized APP in the brain (Figure 1L). A small number of A β_{43} deposits were found in the piriform cortex of 13-month-old mice, and the numbers of A β_{43} deposits were increased in the parietal association cortex and hippocampus of 17-month-old mice (Figure 1, B and C). In 21-month-old mice, extensive A β_{43} -immunopositive plaques appeared in the parietal association cortex, hippocampus, piriform cortex, and striatum (Figure 1D). We further examined A β_{43} and A β_{42} deposition in J20 mice, which express human APP with Swedish and Indiana mutations.¹³ A β_{43} plaques were detected in the hippocampus of 8-month-old mice and these were increased in the hippocampus of 14- and 20-month-old J20 mice in an age-dependent manner (Figure 1, E–G and K). A β_{42} plaques also appeared in the hippocampus of 8-month-old mice and exhibited a pattern similar to that of A β_{43} plaques (Figure 1, H–J).

Deposition of A β_{43} Is More Extensive and Occurs Earlier than A β_{40} in the Brain of APPtg Mouse

By using mouse-derived anti-A β_{40} and rabbit-derived anti-A β_{43} antibodies, A β_{40} and A β_{43} plaques could be visualized separately in the same brain section. The sensitivities of the anti-A β_{40} and anti-A β_{43} antibodies to their antigens are similar.⁷ We then determined the relationship between A β_{43} and A β_{40} in the amyloid plaques in the brain of young and aged APPtg mice. Amyloid plaques in brain parenchyma can be morphologically classified into three different types: diffuse, immature, and mature plaques.¹⁴ In 8-month-old J20 mouse brains, we found diffuse plaques with a majority of A β_{43} immunoreactivity and with very weak A β_{40} immunoreactivity at the center of the plaques. The A β fibrils showing a tendency toward expansion at the edge of the diffuse plaques are A β_{43} immunopositive, but not A β_{40} immunopositive (Figure 2, A–C). Using laser-scanning confocal microscopy, we also found that A β_{43} is prone to be localized in the core of mature plaques in 20-month-old J20 mice (Figure 2D). Because A β_{40} is not the major species depositing in the J20 mouse brain,¹⁵ we then immunostained brain sections from Tg2576 mice. A β_{43} and A β_{40} double

Figure 3 Aβ₄₃ deposits earlier than Aβ₄₂ in diffuse plaques and at neuronal membrane. **A–M, O–Q:** Brain sections from 8-month-old J20 mice (**A–C**) and 13- and 21-month-old Tg2576 mice (**E–M**) were stained for Aβ₄₂ and Aβ₄₃ deposition with anti-Aβ₄₂ monoclonal and anti-Aβ₄₃ polyclonal antibodies. Diffuse plaques showing Aβ₄₃ immunoreactivity, but not Aβ₄₂ immunoreactivity, were found in the hippocampus of 8-month-old J20 mice (**A–C**). Orthogonal views also showed that Aβ₄₃ is the major component and is localized at the center of the diffuse plaques (**C**), and Aβ₄₃ diffuse plaques were greater in number than Aβ₄₂ diffuse plaques in the hippocampus of 8-month-old J20 mice (**D**). Most of Aβ₄₃ plaques colocalized with Aβ₄₂ deposition in the diffuse and mature plaques at a low magnification (**H–J**); however, Aβ₄₃-immunoreactive, but not Aβ₄₂-immunoreactive, Aβ deposition (**arrowheads**) was found on the neuronal membrane or in the neurons (**E–G, K–M, and O–Q**). **N:** Neurons with Aβ₄₃ deposition were greater in number than those with Aβ₄₂ deposition in the cortex of 21-month-old Tg2576 mice. Data are expressed as means ± SEM. *n* = 4 or 8. **P* < 0.05, ***P* < 0.01 Bonferroni–Dunn test. Scale bars: **A–C**, 5 μm; **E–G, K–M, and O–Q**, 10 μm; **H–J**, 50 μm.

immunostaining revealed that Aβ₄₀ deposited in the mature or compact plaques, whereas Aβ₄₃ extensively deposited in the diffuse plaques and in the core of the mature plaques (Figure 2, E–G and J–L). Most of the diffuse plaques with a diameter of less than 20 μm exhibited only Aβ₄₃ immunoreactivity. Interestingly, Aβ₄₀ appeared in the center of some Aβ₄₃-immunoreactive immature plaques (Figure 2, O–Q).

Taken together with the results from 8-month-old mice, these findings suggest that Aβ₄₃ deposits may appear before Aβ₄₀ deposits and that Aβ₄₃ in plaques may be truncated at the C-terminus by protease and converted to Aβ₄₀. The Aβ₄₃ plaque number is 2.2-fold higher than that of Aβ₄₀ in hippocampus and 3.5-fold higher in neocortex (Figure 2R). Differential interference contrast (DIC) microscopy revealed that mature plaques directly disrupted the normal brain structure and that neurons were not present in the mature plaques (Figure 2, H, I, M, and N). In the corpus callosum, the bundle of neural fibers was physically separated and compressed by mature plaques, suggesting the disruption of contralateral axonal projections (Figure 2, H and I). The neurons classified by morphology by DIC microscopy were confirmed by NeuN immunostaining, with greater than 90% accuracy (Supplemental Figure S1). Some portions in a mature

plaque with a diameter greater than 100 μm did not show Aβ₄₀ or Aβ₄₃ immunoreactivity, although the amyloid morphology was clearly observed under DIC microscopy (Figure 2, J–N). This may be accounted for by degradation of Aβ into smaller peptides and loss of the immunoreactivity to Aβ antibody.

Aβ₄₃ Deposits in the Brain Earlier than Aβ₄₂

Aβ₄₂, but not Aβ₄₀, is essential for parenchymal and vascular amyloid deposition in mice.¹⁶ To examine whether Aβ₄₃ deposits more readily and extensively than Aβ₄₂ in the brain, we double-immunostained brain sections from 8-month-old J20 mice using mouse-derived anti-Aβ₄₂ and rabbit-derived anti-Aβ₄₃ antibodies. Interestingly, in the hippocampus of 8-month-old J20 mice, we found some diffuse plaques with most parts exhibiting only Aβ₄₃ immunoreactivity (Figure 3, A–C). Orthogonal views also revealed that Aβ₄₃ is the major component and at the center of the diffuse plaques (Figure 3C). Diffuse plaques exhibiting only Aβ₄₂ immunoreactivity were not found in any of the examined mice, irrespective of age. These results indicate that Aβ₄₃ plaques form earlier than Aβ₄₂ plaques. Aβ₄₃ plaques in the hippocampus of 8-month-old J20 mice were significantly greater in number than Aβ₄₂ plaques (Figure 3D).

In 13-month-old Tg2576 mice, small A β_{43} deposits, but not A β_{42} deposits, were found around the neurons (Figure 3, E–G). In 21-month-old Tg2576 mice, A β_{43} were found colocalized very well with A β_{42} in all diffuse and mature plaques examined at low magnification, suggesting that the anti-A β_{43} and anti-A β_{42} antibodies bind to their antigens with similar affinity (Figure 3, H–J). At high magnification, however, some amyloid deposits on neurons (possibly at the membrane, or perhaps intracellular) exhibited only A β_{43} immunoreactivity, and amyloid deposits exhibiting only A β_{42} immunoreactivity were not found (Figure 3, K–M). Neurons with small A β_{43} deposits are greater in number than those with A β_{42} deposits in 21-month-old mice (Figure 3N). The finding that A β_{43} deposits on the neurons was further confirmed by A β_{43} and NeuN double immunostaining (Figure 3, O–Q). These results imply that A β_{43} forms diffuse plaques and deposits on neurons earlier and more readily than A β_{42} , and that A β_{43} deposition may start from the neurons. A β_{40} , A β_{42} , and A β_{43} are the three species confirmed in amyloid plaques. Our results suggest that A β_{43} may be the earliest-depositing species in the brain.

A β_{43} Level Increases in the Serum and Decreases in the CSF of AD Patients

Because reduced A β_{42} levels and A β_{42} /A β_{40} ratio in plasma and CSF are associated with cognitive decline and the onset of AD,^{17,18} it is crucial to determine whether A β_{43} levels change in AD patients. We measured A β_{43} levels in the serum and CSF of AD patients and age-matched normal controls. Interestingly, serum A β_{43} levels were increased 1.3-fold in AD patients (Figure 4A), but serum A β_{42} levels were significantly decreased, compared with normal subjects (control, 17.5 ± 1.8 pmol/L AD; AD, 10.2 ± 1.1 pmol/L; $P < 0.001$). Serum A β_{40} levels did not differ between the AD and control groups. These changes in A β_{43} and A β_{42} levels resulted in a 2.3-fold increased A β_{43} /A β_{42} ratio in the serum of AD patients, compared with normal subjects (Figure 4C). The serum A β_{43} /A β_{40} ratio did not differ between the control and AD groups (Figure 4E). In accord with the serum findings, CSF A β_{42} levels decreased slightly in AD patients (182.6 ± 8.5 pmol/L), compared with control subjects (224.4 ± 41.5 pmol/L, $P = 0.17$). CSF A β_{43} levels also exhibited a tendency to decrease in AD patients (Figure 4B). In contrast to the serum A β_{43} /A β_{42} ratio, the CSF A β_{43} /A β_{42} ratio exhibited a tendency to decrease in AD patients (Figure 4D). CSF A β_{40} levels in AD patients exhibited a significant 26% increase, compared with control subjects (control, 649.5 ± 70.5 pmol/L; AD, 821.1 ± 24.5 pmol/L) ($P < 0.05$). These changes in A β_{43} and A β_{40} levels resulted in a 60% decreased A β_{43} /A β_{40} ratio in the AD group, compared with the control group (Figure 4F).

ACE Converts A β_{43} to A β_{41} , and This Activity Requires Two Active Domains of ACE

ACE has the activity of converting the longer species A β_{42} to the shorter species A β_{40} , and may regulate A β_{42} /A β_{40}

Figure 4 Higher A β_{43} levels and A β_{43} /A β_{42} ratio in the serum of AD patients. **A** and **B**: Serum and CSF A β levels were determined using A β_{40} , A β_{42} , and A β_{43} ELISA kits. Serum A β_{43} levels in AD patients were higher than those in normal subjects (**A**), whereas CSF A β_{43} levels exhibited a tendency to decrease in AD patients (**B**). **C** and **D**: Serum A β_{43} /A β_{42} ratio in AD patients was higher than normal subjects (**C**) and CSF A β_{43} /A β_{42} ratio exhibited a tendency to decrease in AD patients (**D**). **E** and **F**: Serum A β_{43} /A β_{40} ratio did not differ between normal and AD groups (**E**), whereas CSF A β_{43} /A β_{40} ratio in AD patients was lower than normal subjects (**F**). Data are expressed as means $\pm$ SEM. $n = 18$ to 26 (serum); $n = 6$ (CSF). * $P < 0.05$, ** $P < 0.01$ two-tailed Student's t -test.

ratio in plasma and CSF.⁸ The A β_{42} -to-A β_{40} converting activity of ACE is localized in the N-terminal active domain and its angiotensin-converting activity is predominantly localized in the C-terminal active domain.¹¹ Given the above findings and given that higher blood ACE activity is associated with later onset of AD,¹⁹ we hypothesized that ACE may also convert or degrade A β_{43} and that the increase in A β_{43} levels in AD serum may result from decreased ACE activity or decreased A β_{42} -to-A β_{40} converting activity. Interestingly, we found a decreased A β_{42} -to-A β_{40} converting activity in AD serum, compared with control serum, although ACE activity in AD serum did not differ from the control serum.

Figure 5 Two active domains of ACE are essential for converting Aβ₄₃ to Aβ₄₁. **A**: Schematic representation of the human ACE and recombinant ACE proteins. The wild-type ACE protein contains a signal peptide (SP), a single transmembrane domain (TM), and two homologous catalytic domains. The recombinant ACE proteins F-ACE, N-ACE, C-ACE, F-ACE-E362D, and F-ACE-E960D contain six-histidine residues at the C-terminus and a signal peptide at the N-terminus. **B**: Western blotting and silver staining of 0.1 μg ACE proteins isolated from the culture medium. **C**: 0.5 μmol/L ACE proteins were incubated with Aβ₄₂ or Aβ₄₃. F-ACE and N-ACE with N-domain activity converts Aβ₄₂ to Aβ₄₀, whereas none of the ACE proteins convert Aβ₄₃ to Aβ₄₀. **D**: MALDI-TOF-MS analysis for Aβ₄₃ degradation by F-ACE, N-ACE, or C-ACE. Aβ₄₃ (80 μmol/L) was incubated with 0.5 μmol/L purified F-ACE, N-ACE, or C-ACE at 37°C for 4 hours. Only F-ACE converted Aβ₄₃ to Aβ₄₁. **E**: MALDI-TOF-MS analysis for Aβ₄₃ degradation by the mixture of N-ACE and C-ACE, F-ACE-E362D or F-ACE-E960D.

ACE is a dipeptidyl carboxypeptidase, but it also has endopeptidase activity. To determine whether ACE converts Aβ₄₃ to a shorter species, we generated five recombinant ACE proteins. The N-domain active site in F-ACE was inactivated by mutating glutamate 362 to aspartate (F-ACE-E362D), and the C-domain active site was similarly inactivated by mutating glutamate 960 to aspartate (F-ACE-E960D) (Figure 5A).¹¹ These secreted ACE proteins were isolated from the culture medium by metal ion affinity chromatography and were confirmed by both Western blotting and silver staining (Figure 5B). Purified ACE proteins showed single bands, suggesting the high purity of these proteins. Consistent with our previous findings, ACE activity was predominantly found in ACE proteins with two active domains (F-ACE) or with C-terminal active domain (C-ACE and F-ACE-E362D), but not in ACE proteins with only N-terminal active domain (N-ACE and F-ACE-E960D) (data not shown). To determine whether ACE converts Aβ₄₃ to Aβ₄₀, we incubated Aβ₄₃ with F-ACE, N-ACE, or C-ACE and examined the generation of Aβ₄₀ from

Aβ₄₃ by Western blot using anti-Aβ₄₀ specific antibody. The incubation of Aβ₄₂ with each ACE protein serves as a positive control. F-ACE and N-ACE, but not C-ACE, converted Aβ₄₂ to Aβ₄₀, whereas none of the three ACE proteins could convert Aβ₄₃ to Aβ₄₀ (Figure 5C).

We then performed mass spectrometry analysis to examine the products generated by each ACE protein from Aβ₄₃. A peak corresponding to Aβ₁₋₄₁ was detected in the F-ACE-digested sample, indicating that F-ACE converts Aβ₄₃ to Aβ₄₁ by its dipeptidyl carboxypeptidase activity. Peaks corresponding to Aβ₁₋₃₃, Aβ₁₋₂₈, and Aβ₁₋₂₄ were also generated by F-ACE from Aβ₄₃, which are the same peaks previously found in the sample of Aβ₄₂ digested by ACE, suggesting that the endopeptidyl cleavage of Aβ₄₃ and Aβ₄₂ by ACE occurs at the same sites (Figure 5D).¹¹ Surprisingly, neither N-ACE nor C-ACE exhibited this Aβ₄₃-to-Aβ₄₁ converting activity, suggesting that two active domains are required for converting Aβ₄₃ to Aβ₄₁. Alternatively, at least one active domain is required for the conversion

and the other domain is required to maintain the conformation (Figure 5D). We examined the mixture of N-ACE and C-ACE and found that separated active N-ACE and C-ACE were not able to generate A β ₄₁ from A β ₄₃ (Figure 5E).

Because the truncation may cause a conformational change in N-ACE or C-ACE, thereby causing change in their activities, we examined whether F-ACE-E960D and F-ACE-E362D convert A β ₄₃ to A β ₄₁. Although F-ACE-E960D and F-ACE-E362D generated A β _{1–33}, A β _{1–28}, and A β _{1–24} from A β ₄₃, they failed to convert A β ₄₃ to A β ₄₁ (Figure 5E). These results suggest that both the N- and C-domain of ACE have the endopeptidase activity to generate A β _{1–33}, A β _{1–28}, and A β _{1–24}; however, the A β ₄₃-to-A β ₄₁ converting activity requires two active domains of ACE.

Inhibition of ACE Enhances Brain A β ₄₃ Deposition

No A β ₄₁ was in amyloid plaques of AD patients,⁵ suggesting that A β ₄₁ may not be an amyloidogenic species and that the conversion of A β ₄₃ to A β ₄₁ by ACE may prevent A β ₄₃ deposition. To determine whether ACE affects A β ₄₃ deposition in the brain of APPtg mouse, we administered the ACE inhibitor captopril to Tg2576 mice. Tg2576 mice were fed with a captopril-supplemented diet for 11 months from 6 months of age. At 17 months of age, control Tg2576 mice had an average of 10 A β ₄₃ plaques in the hippocampus and 22 A β ₄₃ plaques in the cortex per sagittal brain section. In contrast, captopril-treated Tg2576 mice had threefold more A β ₄₃ plaques in hippocampus and cortex (Figure 6, A and B). Captopril treatment decreased ACE activity in the cortex by 26% in the 17-month-old captopril-treated Tg2576 mice, compared with control mice. The number of A β ₄₃ plaques in the cortex was significantly and inversely correlated with decreased ACE activity (Figure 6C). These results indicate that inhibition of ACE activity increases A β ₄₃ deposition.

Discussion

Abnormal accumulation of A β is considered a key pathogenic mechanism in AD. Currently, three A β species (A β ₄₀, A β ₄₂, and A β ₄₃) have been identified as accumulating in amyloid plaques of human brain from sporadic or rare familial AD cases.⁵ Compared with A β ₄₀, A β ₄₂ is more neurotoxic, and it seems to be of particular importance for plaque formation.^{2,16} A β ₄₃ has an aggregation propensity similar to or stronger than that of A β ₄₂,^{5,7} but little is known about whether A β ₄₃ deposition differs from A β ₄₂ deposition in the brain, nor about what processes regulate A β ₄₃ deposition. In the present study, using two strains of APP transgenic mice, we found that A β ₄₃ deposition may precede A β ₄₀ and A β ₄₂ deposition in the brain. In addition, we found that ACE with two active domains converts A β ₄₃ to A β ₄₁ and that inhibition of ACE significantly enhances brain A β ₄₃ deposition. These results suggest that A β ₄₃ is a pivotal species other than A β ₄₂ in the early pathogenesis of AD without presenilin mutations. The conversion and

Figure 6 Inhibition of ACE leads to increased brain A β ₄₃ deposition. **A:** Sagittal brain sections from control and captopril-treated Tg2576 mice were stained with anti-A β ₄₃ antibody. A β ₄₃-immunoreactive deposits significantly increased in the cortex and hippocampus of captopril-treated mice, compared with control mice. Representative images are shown. **B:** Quantitation of A β ₄₃-immunoreactive plaque number in hippocampus and cortex. **C:** Significant inverse correlation between ACE activity and the number of A β ₄₃-immunoreactive plaques in cortex. Data are expressed as means $\pm$ SEM. $n = 11$ to 15. ** $P < 0.01$ Bonferroni–Dunn test (**B**) or $P = 0.0029$ Spearman’s rank test (**C**). Scale bar = 500 μ m.

degradation of A β ₄₃ by ACE seems to be important for reducing brain A β ₄₃ deposition.

A β ₄₃ deposition in the brain of APP transgenic mice was more extensive and occurred earlier than A β ₄₀ deposition. A β ₄₃ colocalized well with A β ₄₂ in core plaques and diffuse plaques of aged APP transgenic mice. However, A β ₄₃ were found more frequently than A β ₄₂ deposits, and were localized exclusively on the neuronal body; most neuronal A β deposits exhibited only A β ₄₃ immunoreactivity (Figure 3), suggesting that A β ₄₃ plaques could start from the neurons. GM1 ganglioside-bound A β ₄₂, but not A β ₄₀, found in AD brains has been suggested to be a seed for amyloidogenesis.²⁰ Whether A β ₄₃ binds to GM1 ganglioside on membrane and whether an A β ₄₃–GM1 complex could also serve as a seed for amyloidogenesis remains to be elucidated. Diffuse plaques and small A β deposits exhibiting only A β ₄₃ immunoreactivity were identified in J20 mice at 8 months of age and in Tg2576 mice at 13 months of age, but diffuse plaques or small A β deposits exhibiting only A β ₄₂ immunoreactivity could not be found. These lines of evidence indicate that A β ₄₃ deposits earlier than A β ₄₂ at the early stage when amyloid plaques start to appear. Thus, the order of appearance of parenchymal amyloid plaques might be A β ₄₃-immunoreactive plaques first, then A β ₄₂-immunoreactive plaques, and A β ₄₀-immunoreactive plaques last of

all. Because A β ₄₀ transgenic mice do not form amyloid plaques, the A β ₄₀-immunoreactive plaques may derive from conversion of A β ₄₃- or A β ₄₂-immunoreactive plaques.^{2,8} The conversion of injected A β ₄₂ plaques to A β ₄₀-immunoreactive plaques was confirmed in mouse brain.²¹ Alternatively, the early-appearing A β ₄₃ plaques may serve as seeds and trigger the accumulation of A β ₄₂ and A β ₄₀.

The two longer species A β ₄₂ and A β ₄₃ are highly amyloidogenic and neurotoxic. In contrast, the shorter species A β ₄₀ inhibits amyloid plaque formation and inhibits the neurotoxicity of the A β ₄₂.^{2,22} Thus, the conversion of a longer A β species to a shorter species emerges as a therapeutic strategy for AD treatment.^{4,23} The γ -secretase modulators are such candidates, which can shift the γ -cleavage from producing longer A β species to shorter species. However, targeting γ -secretase always has the associated difficulties of avoiding changing the processing of Notch and other important γ -substrates.³ Instead of modulating γ -secretase, ACE (with its active sites in the extracellular space) could convert the secreted longer species A β ₄₂ to a shorter species, A β ₄₀.⁸ This may reduce the level of toxic A β ₄₂ and the A β ₄₂/A β ₄₀ ratio and increase neuroprotective A β ₄₀. The conversion of A β ₄₂ to A β ₄₀ may occur in the blood and CSF, and in the extracellular space of the brain, without changing the processing of Notch and other γ -substrates.

Here, we have shown that ACE converts A β ₄₃ to A β ₄₁ and that this activity requires two active domains of ACE (Figure 5). Naturally occurring N-domain of human ACE was found in ileal fluid,²⁴ and testis ACE contained only the C-domain of ACE,²⁵ suggesting that the three forms of ACE containing either or both of the N- and C-domains may have their specific substrates. For the first time, we demonstrate that the two active domains of ACE need to collaborate to convert A β ₄₃ to A β ₄₁, whereas N-domain of ACE is sufficient to convert A β ₄₂ to A β ₄₀.¹¹ ACE containing either one or both active domains may have a different confirmation, which governs how ACE accesses its substrates. Although whether the A β ₄₁ species has neurotoxicity or anti-amyloid effects needs further study, A β ₄₁ is likely to be much less neurotoxic and amyloidogenic than A β ₄₂ or A β ₄₃ because it has not been found in the amyloid plaques. The finding that inhibition of ACE activity, which converts A β ₄₃ to A β ₄₁, enhances A β ₄₃ deposition supports this notion.

In conclusion, our findings suggest that A β ₄₃ is the earliest-depositing species in the brain of APPtg mouse. A β ₄₃ plaque formation does not necessarily involve the particular *PSEN1* mutations that cause overproduction of A β ₄₃.⁷ Similar to the conversion of A β ₄₂ to A β ₄₀ by ACE, A β ₄₃ can be processed by ACE at its C-terminal and converted to A β ₄₁. Inhibition of ACE activity significantly enhanced brain A β ₄₃ deposition. Because the shorter A β species might have a protective effect against the neurotoxicity of the longer species,^{2,26} C-terminal conversion of a longer-form to a shorter form of A β species by ACE or other carboxypeptidases may serve as a novel strategy for anti-amyloid therapy.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.01.053>.

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