β-Amyloid Racemized at the Ser26 Residue in the Brains of Patients with Alzheimer Disease:
Implications in the Pathogenesis of Alzheimer Disease

TAKEKAZU KUBO, PhD, YOSHIHIRO KUMAGAE, PhD, CAROL A. MILLER, MD, AND ISAO KANEKO, PhD

Abstract. Oligomeric and fibrillar β-amyloid (Aβ) may be toxic in Alzheimer disease (AD), especially after post-translation modification cumulative over time. Racemization of Ser and Asp residues of Aβ in senile plaques (SPs) occurs as an age-dependent process in AD. We previously reported that Aβ1–40 racemized at Ser26 is soluble and susceptible to proteolysis yielding toxic [D-Ser26]Aβ25–35/40 fragments in vitro and in vivo. Here, we focus on the localization of racemized Ser26 residues in AD brains within the limbic system, the earliest site of AD histopathology. We developed antisera (20.1 and 22.7), each with epitopes within [D-Ser26]Aβ25–40. Two forms of truncated [D-Ser26]Aβ were detected either in SPs or within neurons in all 11 AD-affected brains, but not in age-matched controls. [D-Ser26]Aβ25/26–35 (detected by 20.1) was localized to plaque cores, extracellular neurofibrillary “ghost” tangles and vascular amyloid deposits. In contrast, [D-Ser26]Aβ25–40 (detected by 22.7) was observed in most neurons containing intracellular neurofibrillary tangles, but not in SPs. These results suggest [D-Ser26]Aβ1–40, formed during aging, becomes soluble and diffuse from SPs. It is then proteolyzed to [D-Ser26]Aβ25–35/40, which is toxic and may contribute to the neurodegeneration. This hypothesis may explain the long lag between SP formation and neurofibrillary degeneration in AD brains.

Key Words: Aging; D-Ser; Hippocampus; Neurodegeneration; Neurofibrillary tangle; Racemization; Senile plaque.

INTRODUCTION

β-Amyloid (Aβ) is the principal component of senile plaques (SPs). Neurotoxicity of fibrillar Aβ in vitro has been reported by many investigators (1–3) as a major contributor to AD pathogenesis (4–7). We previously demonstrated that fibrillar Aβ produces dramatic neurodegeneration in vivo by enhancing the susceptibility of neurons to excitatory amino acids (8, 9). However, the temporal and topographical dichotomy between the appearance of SPs and the neurofibrillary degeneration in AD remains unresolved. It is unclear if insoluble, fibrillar Aβ per se is directly responsible for the neurodegeneration (10, 11). Initially, Aβ deposits appear as diffuse plaques 20 to 30 years prior to the neurofibrillary degeneration in Alzheimer disease (AD) (12, 13). Second, large accumulations of Aβ induce little or no neurodegeneration in brains of transgenic mice (5, 6). Third, the hippocampal CA1 region, and the subiculum, both vulnerable areas early in AD, are more typically spared of Aβ deposition, especially in the early stage (14). Finally, soluble oligomeric forms of Aβ species have been implicated with toxicity linked to AD (15, 16). These observations suggest that a multistep, age-dependent solubilization processing of Aβ may be necessary to induce degeneration in AD patients.

Racemization of Ser and Asp residues especially in insoluble and nonmetabolized proteins is a typical age-dependent process, and is known to be responsible for some age-related diseases in humans (17, 18). Aβ1–40 has 2 Ser (#8, 26) and 3 Asp (#1, 7, 23) residues. Considerable amounts (5%–10%) of D-Ser and D-Asp are present in insoluble Aβ in SPs from AD brains (19–21).

To explore the possible roles of racemization of Aβ in the pathogenesis of AD, we focused on Aβ racemized at the Ser 26 residue ([D-Ser26]Aβ1–40) because [D-Ser26]Aβ1–40 differs from its parent Aβ1–40 both physically and biologically, is non-neurotoxic, soluble and thus capable of being released from SPs, and finally, is converted by brain proteases to a protease-resistant and neurotoxic fragment, [D-Ser26]Aβ25–35/40 in vitro (1, 22, 23).

When injected into the rat hippocampus with a sublethal dose of ibotenic acid, an excitatory amino acid, [D-Ser26]Aβ1–40 produces severe neuronal loss suggesting conversion of nontoxic [D-Ser26]Aβ1–40 into a toxic form such as [D-Ser26]Aβ25–35/40 (22). These observations suggest a series of events initiated by age-dependent racemization of Aβ1–40 in SPs followed by increasing solubility, then release from plaques, and conversion by brain proteases to a toxic form, either [D-Ser26]Aβ25–35 or [D-Ser26]Aβ25–40 in AD (1, 22, 23).

In this study, we investigated the localization of Aβ with a D-Ser26 residue, especially the truncated and toxic fragments, [D-Ser26]Aβ25–35/40 in AD brains by immunohistochemical analysis using antibodies (20.1 and 22.7) specific to [D-Ser26]Aβ25–35/40. Localization of truncated Aβ species racemized at Ser26 present in the cores of SPs and degenerating hippocampal neurons in...
Putative epitopes of 20.1 and 22.7:

20.1

22.7


\[\text{d-Ser}^{20}\text{A}\beta^{25-40}\]


Fig. 2. Truncated Aβ fragments racemized at Ser26 in SPs are detected by antiserum 20.1 in AD brains. On cryosections (10 μm) of hippocampus and amygdala from AD patients (A, C–F) and a control subject (B), immunohistochemistry was performed with antiserum 20.1 (A–C, E, F). Antigen retrieval with formic acid was performed prior to staining (A, B, E, F), and (D) is stained with thioflavin S. Antiserum 20.1 stained many circumscribed foci suggestive of SPs, seen in red, in the amygdala (A) of an AD brain (AD-7) and subiculum (C) of another AD brain (AD-4) but had no immunoreactivity in the amygdala (B) of the control brain (Control-4). Preimmune serum had no immunoreactivity in AD brains (data not shown). At higher magnification, the 20.1-positive SP (C) in the subiculum of AD (AD-4) was also stained with thioflavin S (D) in a serial section. Racemized and truncated Aβ was further confirmed after immunosorption (E, F) whereby immunostaining of the amygdala from AD brain (AD-7) was performed following preincubation of 20.1 with [D-Ser26]Aβ25-35 (25 ng/ml) (E) or Aβ25-35 (25 ng/ml) (F). Immunoreactivity of 20.1 in SPs was completely blocked by [D-Ser26]Aβ25-35 (E), but not by the L-form of Aβ25-35 (F) or racemized longer form of Aβ, [D-Ser26]Aβ1–40 (not shown). Sections were counterstained with hematoxylin. Scale bar = 100 μm.

AD-affected brains and in age-matched control brains was examined.

MATERIALS AND METHODS

Subjects

Human brain tissues were obtained postmortem from the Neuropathology Core of the Alzheimer’s Disease Research Center (ADRC) of the University of Southern California. All procedures were carried out with strict adherence to the guidelines of Sankyo Ethical Committee for the usage of human samples. Tissue blocks (1 cm³) of hippocampus, including entorhinal cortex and amygdaloid nucleus, were dissected from brain...
regions at autopsy, snap-frozen in liquid nitrogen-chilled isopentane (Sigma-Aldrich, St. Louis, MO), and stored at −80°C until use. These sites were selected as they have the earliest pathology in AD, and thus most likely to have racemized Aβ. Included were 11 clinically and neuropathologically confirmed AD patients and 7 control subjects, including 2 amyotrophic lateral sclerosis (ALS) patients without either a clinical history of dementia or AD-related neuropathological changes (Table 1). The ages of AD patients and control subjects were 75.5 and 73.6 years, respectively. Mean postmortem intervals were 4.5 hours (h) (range 3 h–8 h) for AD and 6.5 h (range 4 h–12 h) for controls. The number of neuritic plaques and vascular amyloids in the entorhinal cortex and those of NFTs in hippocampal CA1 were semiquantitatively scored as follows: − = none, + = sparse, ++ = moderate, +++ = frequent. In addition, the Braak stage of each case was determined by grading scheme shown in reference 14 and 25.

Abbreviations: PMD, postmortem delay; F, female; M, male; ALS, amyotrophic lateral sclerosis.

* This case had sparse neuritic plaques but numerous diffuse plaques.

Peptides and Antibodies

Human Aβ peptides containing D-amino acids were synthesized as previously described (1) and Aβ25–35 or Aβ1–40 was purchased from Sigma-Aldrich. Rabbit polyclonal anti-[D-Ser25]Aβ25–35 antisera (20.1 and 22.7) were generated by immunizing rabbits with synthetic, racemized Aβ peptides conjugated with keyhole limpet hemocyanin (KLH). Amino acid sequences of peptide immunogens were Gly-D-Ser-Asn-Lys-Gly-ala-Cys for 20.1 and Gly-D-Ser-Asn-Lys-Gly-D-Ser-Asn-Lys-Gly-D-Ser-Asn-Lys-Cys for 22.7. The 2 sequences correspond to [D-Ser25]Aβ25–30 and 3 repeats of [D-Ser25]Aβ25–28, with a cysteine at the carboxyl terminus, respectively. Synthesis of peptide immunogens, immunization of rabbits, and collection of antisera was performed at Asahi Techno Glass Corporation (Tokyo, Japan). Aliquots of antisera as well as preimmune sera were frozen at −80°C. We used whole antisera (20.1 and 22.7) in the experiments, but each purified rabbit IgG obtained by using a protein-A conjugated agarose column (Bio-Rad Laboratories, Hercules, CA) showed the same staining patterns (data not shown). Anti-Aβ antibodies, 6E10 and 4G8, recognizing amino acids 1–17 and 17–24 of Aβ, respectively, were purchased from Senetek (Maryland Heights, MO). Polyclonal rabbit anti-Aβ40 and Aβ42 C-terminal antibodies were obtained from Biosource International, Inc. (Camarillo, CA). AT-8 (Innogenetics, Ghent, Belgium) was used to visualize hyperphosphorylated tau. Anti-amyloid precursor protein (APP) antibody, 22C11, and anti-tau antibody, tau-1, were purchased from Research Diagnostics (Flanders, NJ) and Roche Diagnostics (Mannheim, Germany), respectively.

Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

Microtiter plates (Sumitomo Bakelites, Tokyo, Japan) were coated with 20 μg/ml of [D or L-Ser25]Aβ25–35 or [D or L-Ser25]Aβ1–40 in 0.1 ml of 100 mM NaHCO3 (Sigma-Aldrich) buffer, pH 9.6, at 4°C overnight. After washing, the plates with phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA) containing 0.05% Tween 20 (Sigma-Aldrich), unoccupied binding sites were blocked with PBS containing 0.05% Tween 20 and 3% bovine serum albumin (Sigma-Aldrich) at room temperature.
for 3 h. Antiserum of 100 μl (20.1 or 22.7) diluted 100-fold was then applied in triplicate and incubated at room temperature for 1 h. After washing 3 times with PBS containing 0.05% Tween 20, a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG, Sigma-Aldrich) was added at a 1,000-fold dilution in 0.1 ml of PBS and incubated at room temperature for 1 h. Bound enzyme activities were measured by TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate kit (Sumitomo Bakelite). For competitive binding experiments, antiserum (20.1 or 22.7) diluted at 100-fold was preincubated with competitive peptides for 2 h at room temperature before application to the plates coated with [D-Ser26]Aβ25–35 as described above.

Immunohistochemistry

Cryostat sections (10 μm) were placed onto slides coated with polylysine (Matsunami Glass, Osaka, Japan). Unless otherwise mentioned, antigen retrieval treatments of sections were performed. Sections were fixed with formaldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 30 min, treated with 98% to 99% formic acid (Sigma-Aldrich) for 10 min at room temperature, incubated in 3% hydrogen peroxide (Sigma-Aldrich) in methanol (Wako Pure Chemical Industries) for 30 min, then in 10% normal goat serum (Chemicon International, Temecula, CA) in PBS for 2 h at room temperature to block endogenous peroxidase activity and nonspecific staining, respectively. Thereafter, sections were reacted with primary antibodies in PBS at 4°C overnight. Antibodies (20.1 and 22.7) were typically used at a 200-fold dilution. Sections were then positively stained with 20.1 and 22.7 by microwave-boiling the sections at 90°C for 2 h, and then probed with 200-fold dilutions of either [D-Ser26]Aβ25–35 antisera (20.1 and 22.7) with other AD-related molecules by Western blotting of AD and control brain homogenates. Frozen hippocampal sections (10 μm) of AD or control subjects were directly lysed in SDS sample buffer (31 mM Tris-HCl (pH 6.8, Sigma-Aldrich), 1% SDS (Bio-Rad Laboratories), 12.5% glycerol (Sigma-Aldrich), 0.005% Bromophenol Blue (Bio-Rad Laboratories) and 1% β-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min. Aliquots (30 μg) of proteins of each mixture were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skim milk (Bio-Rad Laboratories) in Tris-saline (pH 7.4) at room temperature for 2 h, and then probed with 200-fold dilutions of either [D-Ser26]Aβ25–35 antisera (20.1 and 22.7), anti-APP antibody (2C11), anti-tau antibody (tau-1) or anti-hyperphosphorylated tau antibody (AT-8) at 4°C overnight. The membranes were then reacted with a secondary antibody (Transduction Laboratories) conjugated with HRP at room temperature for 1 h and developed using an ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Western Blotting

We checked the cross-reactivity of anti-[D-Ser26]Aβ25–35 antibodies (20.1 and 22.7) with other AD-related molecules by Western blotting of AD and control brain homogenates. Frozen hippocampal sections (10 μm) of AD or control subjects were directly lysed in SDS sample buffer (31 mM Tris-HCl (pH 6.8, Sigma-Aldrich), 1% SDS (Bio-Rad Laboratories), 12.5% glycerol (Sigma-Aldrich), 0.005% Bromophenol Blue (Bio-Rad Laboratories) and 1% β-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min. Aliquots (30 μg) of proteins of each mixture were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skim milk (Bio-Rad Laboratories) in Tris-saline (pH 7.4) at room temperature for 2 h, and then probed with 200-fold dilutions of either [D-Ser26]Aβ25–35 antisera (20.1 and 22.7), anti-APP antibody (2C11), anti-tau antibody (tau-1) or anti-hyperphosphorylated tau antibody (AT-8) at 4°C overnight. The membranes were then reacted with a secondary antibody (Transduction Laboratories) conjugated with HRP at room temperature for 1 h and developed using an ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RESULTS

Specificities of Anti-[D-Ser26]Aβ25–35 Antibodies (20.1 and 22.7)

We previously hypothesized that Aβ racemized at Ser26 plays an important role in the pathogenesis of AD (22, 23). In the present study, we developed 2 polyclonal antisera, (20.1 and 22.7) specific to [D-Ser26]Aβ25–35/40 to prove the existence of [D-Ser26]Aβ in brains of AD patients. The specificity of 20.1 or 22.7 was determined by ELISA (Fig. 1A, B). Both antisera bound in a dose-dependent fashion to [D-Ser26]Aβ25–35, but neither to Aβ25–35 nor Aβ1–40 coated on the plastic plates, indicating essentially no cross-reactivity of the antisera with either Aβ25–35 or Aβ1–40, each of which contained an L-Ser26 residue. Further, [D-Ser26]Aβ1–40 was also not

Fig. 3. Localization of 20.1-positive Aβ-fragment in the cores of senile plaques. Cryosections from the amygdala (A, B) and entorhinal cortex (C–E) of AD brains (AD-7 and AD-5, respectively) were pretreated with formic acid for antigen retrieval and then sequentially stained with 20.1 and either anti-β-amyloid antibody, 4G8 (A, E) or anti-β-amyloid antibody, 6E10 (B). Immunostaining with 20.1 is shown in red (A, B, D, E) and that with 4G8 (A, C, E) or 6E10 (B) is shown in green. The colocalization of 20.1 with 4G8 or 6E10 immunoreactivity appears in yellow. Note that most of the 20.1-positive plaques were labeled with 4G8 but not with 6E10. C: Senile plaques with or without a core shown with the Oregon green (4G8) filter. D: The same field as shown in (C) is shown with the Texas red (20.1) filter. A merged image of (C) and (D) is presented in panel (E).

recognized by either antiserum. Only [D-Ser26]Aβ25–35, which contains a truncated N-terminal amino acid near the D-Ser residue, was recognized.

To further examine the specificity of these 2 antisera, we performed the competitive experiments in ELISA as follows: Diluted antiserum (20.1 or 22.7) was preincubated with competitive peptides for 2 h before application to plates coated with [D-Ser26]Aβ25–35, and then immunoreactivity determined as described in Materials and Methods. The data shown in Figure 1C clearly indicate that the reactivity of 20.1 with [D-Ser26]Aβ25–35 antigen was completely abolished by preincubation with [D-Ser26]Aβ25–35 or [D-Ser26]Aβ26–35, but not with [D-Ser26]Aβ25–40. On the other hand, the reactivity of 22.7 to [D-Ser26]Aβ25–35 was inhibited by preincubation with [D-Ser26]Aβ25–35 or [D-Ser26]Aβ26–35, but not with [D-Ser26]Aβ26–35 (Fig. 1D). Neither Aβ25–35, Aβ1–40 nor Aβ1–40 with D-Ser6, D-Ser26, D-Asp1, D-Asp7, or D-Asp23 inhibited the reactivity of 20.1 or 22.7. These data suggest that both antisera exhibit highly specific reactivity to truncated [D-Ser26]Aβ25–35, but their precise recognition sites differ: epitope regions of 20.1 and 22.7 were estimated to [D-Ser26]Aβ26–30 and [D-Ser26]Aβ25–29, respectively (Fig. 1E).

Localization of 20.1-Immunoreactivity to Mature Plaques

We investigated whether or not truncated [D-Ser26]Aβ is present in the brains of AD patients by immunohistochemistry with anti-[D-Ser26]Aβ25–35 antisera (20.1 and 22.7). The CNS of AD patients with abundant neuritic plaques and NFTs and control tissues essentially free of neuritic plaques and tangles were compared (Table 1). First, we performed the immunohistochemical analysis with 20.1, which includes the [D-Ser26]Aβ26–30 epitope. SP cores were abundantly stained by 20.1 in the amygdala and entorhinal cortex and, to a lesser extent, in hippocampal subiculum of AD brains (Fig. 2A, C). Corresponding preimmune serum showed no immunoreactivity (data not shown). Plaque cores immunoreactive with 20.1 were detected in the 9 AD brains, all with abundant neuritic plaques in the entorhinal cortex (Table 1).

In contrast, 4G8-positive diffuse amyloid deposits exhibited no immunoreactivity with 20.1 (C–E, arrows). Scale bar = 50 μm.
Fig. 4. Vascular amyloid and extracellular NFTs stained with 20.1 in AD brain. Serial cryosections were obtained from AD hippocampus with amyloid angiopathy and extracellular NFTs. Sections were not treated with formic acid. Each section was stained with 20.1 (A, B, E), 6E10 (C), or thioflavin S (D, F). The 20.1-positive truncated and racemized Aβ was also present in thioflavin S- and 6E10-positive vascular amyloid in the CA1 area (AD-3; A–D) and in the hippocampal CA2 area (AD-4; not shown). The 20.1-positive vessel (arrow) in (A) is presented at higher magnification in (B). The 20.1-positive staining of thioflavin S-positive extracellular NFTs was observed in the CA2 area (AD-4; E, F) and in the CA1 area (AD-10, AD-11; data not shown). Scale bar = 100 μm.

Fig. 5. Antiserum 22.7 showed intense staining in CA1 pyramidal neurons in the AD hippocampus. Hippocampal cryosections including the CA1 region were prepared from 3 AD (AD-11: A, F, G; AD-9: B; AD-5: D, E) and 1 control brain (Control-4: C). All sections except those in (B, D, E) were pretreated with formic acid. Sections were stained with 22.7 (A–D, F, G) or a nuclear dye, Hoechst 33258 (E). In AD hippocampi, 22.7 stains the CA1 neurons with nuclei visualized by hematoxylin (A, B). Double staining with both 22.7 and Hoechst 33258 confirmed a 22.7-positive pyramidal neuron (D) exhibiting a nucleus stained with Hoechst 33258 (E). Arrows indicate the same neuron in photo (D) and (E). For immunosorption, staining was performed by 22.7 preincubated with [D-Ser26]Aβ25–40 (10 μg/ml, F) or Aβ25–40 (10 μg/ml, G). Neuronal staining by 22.7 was considerably abolished by preincubation with [D-Ser26]Aβ25–40, but not with Aβ25–40 or [D-Ser26]Aβ1–40 (not shown). Scale bar = 50 μm.

TABLE 2
Localization of [D-Ser26]Aβ25/26–35/40 Antigens in AD Brains

<table>
<thead>
<tr>
<th>Neurons</th>
<th>Hippocampus</th>
<th>Plaques</th>
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<tr>
<td>Subiculum</td>
<td>CA1</td>
<td>CA2</td>
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<tr>
<td>20.1</td>
<td>–</td>
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<td>22.7</td>
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The typical distribution of 20.1- and 22.7-positive neurons and plaques is summarized. The intensity of the positive staining in each region is represented as follows: – = none, + = sparse, ++ = moderate, +++ = frequent. [D-Ser26]Aβ25–40 antigens labeled with 22.7 were detected mostly within neurons, while [D-Ser26]Aβ26–35 antigens labeled with 20.1 were detected in extracellular structures such as plaques and extracellular NFTs.

* The 20.1-positive plaques in the hippocampus were frequently observed in the subiculum. No significant immunoreactivity of 20.1 or 22.7 was observed in all 7 age-matched controls. Abbreviations: DG, dentate gyrus; Ent. Ctx., entorhinal cortex.
with Aβ25–35 (Fig. 2E, F), [D-Ser²⁶]Aβ25–40 or [D-Ser²⁶]Aβ1–40 (not shown), in keeping with the ELISA competitive binding experiments (Fig. 1C). [D-Ser²⁶]Aβ26–35 also inhibited the 20.1-positive immunostaining (data not shown). These results strongly suggest that the immunopositive plaque cores contain [D-Ser²⁶]Aβ25/26–35-like truncated peptides. Because thioflavin S stained a 20.1-positive deposit in a serial section of an AD (AD-4) brain (Fig. 2C, D), antigens detected by 20.1 were estimated to be associated with mature plaques with amyloid in a β-pleated sheet conformation in AD brains. Semiquantitative analysis demonstrated that 20.1-positive deposits were detected in 87% of thioflavin S-positive plaques (124 of 20.1-positive deposits out of 143 thioflavin S-positive plaques) in amygdaloid serial sections of an AD (AD-7) brain.

Colocalization of 20.1-immunoreactivity with Aβ deposits was clearly shown in a section double labeled with 20.1 and anti-Aβ antibodies, 4G8 (Fig. 3A, E) and 6E10 (Fig. 3B), recognizing amino acids 17–24 and 1–17 of Aβ, respectively. Antibody 4G8 reacted with almost all of the 20.1-positive deposits, and 20.1 reacted with 60% of 4G8-positive plaques (124 of 20.1-positive plaques among 205 of 4G8-positive plaques) (Fig. 3A). In contrast, 6E10 labeled some of the 20.1-positive deposits (Fig. 3B). It should be noted that 20.1 immunoreactivity was concentrated in the plaque cores and surrounded by 4G8 or 6E10-positive Aβ halos (Fig. 3A, B, E). In contrast, 20.1-staining was not observed in 4G8-positive diffuse plaques (Fig. 3C–E, arrows). These results indicate racemized forms of Aβ aggregated within the plaque cores.

We analyzed brains obtained from 2 AD patients (AD-3 and AD-4) with typical amyloid angiopathy (Table 1). The vascular media were also intensely stained by 20.1 and in serial sections, the 20.1-positive vascular deposits also reacted with 6E10 and thioflavin S (Fig. 4A–D), suggesting a β-pleated structure is included in at least some of the Aβ fragments.

Extracellular “ghost tangles” contain 20.1-immunoreactive racemized Aβ. Antibody 20.1-positive NFTs were present in the hippocampal CA1 and CA2 regions of 3 AD (AD-4, AD-10, and AD-11) patients (Fig. 4E, F; Table 2). Although dependent on the section orientation, these 20.1-positive tangles had no associated nucleus and a less compact fibrillar structure suggestive of extracellular or “ghost” tangles (26, 27). In contrast, in tissues of all 10 AD patients (see below), the 20.1 antiserum did not stain the 22.7-positive intracellular NFTs within pyramidal neurons where nuclei were clearly apparent. With regards to both the vascular amyloid and extracellular NFTs, the 20.1-positive staining was also inhibited by [D-Ser²⁶]Aβ25–35 but not by Aβ25–35 or [D-Ser²⁶]Aβ1–40 (data not shown).

Racemized Aβ Detected by 22.7 Localizes to Intracellular NFTs

Next, we performed immunohistochemical analysis with antibody, 22.7, in which the epitope lies within [D-Ser²⁶]Aβ25–29. Figure 5 shows typical immunostaining by 22.7 of neurons with NFT in the AD-affected hippocampal CA1 region and a normal control. Although plaques remained unstained, many, but not all pyramidal neurons in the hippocampal CA1 region were reactive with 22.7. Thioflavin-S-positive pyramidal neurons confirmed localization of the NFTs (not shown). A corresponding preimmune serum exhibited no staining in AD brains (data not shown). In AD-affected brains, similar 22.7-positive neuronal staining was also frequently observed in neurons of the amygdala, entorhinal cortex, and subiculum (data not shown) but not in hippocampal CA3 or dentate granule neurons (Table 2).

Immunoreactivity of 22.7 was detected in the neuronal cytoplasm but not in the nuclei (Fig. 5A, B, D). There was a gradation of immunoreactivity among neurons; 10% to 20% of positive neurons were strongly stained and the remainder moderately or weakly reactive. Glial cells in those areas were not labeled with 22.7. We observed 22.7-positive neurons in all 10 AD brains tested (AD-1, AD-3–AD-11; AD-2 was not tested), but no significant staining in the 7 control brains (Fig. 5C and data not shown). Most of the 22.7-positive neurons were of comparable size to intact, unstained pyramidal neurons, although there were a few larger cells with a ghost tangle-like appearance (Fig. 5A, B, D and data not shown).

Among the 22.7-positive neurons, nuclei were visualized by either hematoxylin (Fig. 5A, B) or by double labeling with 22.7 and Hoechst 33258, a fluorescent nuclear stain (Fig. 5D, E). These results suggest that such intact neurons already contained 22.7-positive and truncated [D-Ser²⁶]Aβ peptides and neurofibrillary changes.

As shown in Figure 5F and G, most 22.7 immunoreactivity was inhibited by preincubation of 22.7 with [D-Ser²⁶]Aβ25–40 (78% inhibition, 101 out of 134 neurons) but not with Aβ25–35, [D-Ser²⁶]Aβ26–35 or [D-Ser²⁶]Aβ1–40 (not shown), as was the case with the competitive ELISA experiment (Fig. 1D). However, in contrast to the ELISA experiments, [D-Ser²⁶]Aβ25–35 did not inhibit the 22.7-positive staining in AD brains (data not shown). A part (22%) of 22.7 staining, which was strong in its intensity, was not completely inhibited even by preincubation with the highest concentrations of [D-Ser²⁶]Aβ25–40 used. These results suggest that most of the 22.7-positive antigens were [D-Ser²⁶]Aβ25–40 peptides rather than [D-Ser²⁶]Aβ25–35. The C-terminal fragments of Aβ1–40, detected by commercially available anti-Aβ40 C-terminal-specific antibody, were accumulated in the hippocampal CA1 neurons and senile plaques.
TRUNCATED Aβ WITH D-SER26 IN NEURONS IN AD BRAIN

(6A). Colocalization of 22.7 antigens with Aβ fragments ending at Val40 in neurons was confirmed by double immunostaining of the same AD sections with 22.7 and anti-Aβ40 C-terminal-specific antibody (Fig. 6D). These results support the idea that some of the 22.7 antigens in neurons are [D-Ser26]Aβ25–40 peptides, although the presence of [D-Ser26]Aβ25–35 and other truncated [D-Ser26]Aβ peptides cannot be excluded in view of the results of the antiserum in the ELISA experiments.

Immunostaining of the CA1 region in AD-affected brain was performed using both 22.7 and AT-8, a monoclonal antibody recognizing hyperphosphorylated tau in degenerating neurons containing NFTs. Although AT-8 stained numerous NFTs, neuritpl threads, and neuritic plaques (Fig. 6B), 22.7 reacted with some of the AT-8-positive neuronal somata, but not AT-8-positive neuritic threads or neuritic plaques (Fig. 6C, E). Colocalization of 22.7-immunoreactivity and abnormal tau in NFTs was confirmed by sequential labeling with 22.7 and AT-8: Some neurons containing AT-8-positive NFTs were also stained with 22.7 (Fig. 6E). By semiquantitative analysis, 22.7-positive neurons were detected in 67% of AT-8-positive neurons (112 of 227-positive neurons out of 168 AT-8-positive neurons) in hippocampal CA1 of an AD (AD-11) brain. These data strongly suggest that [D-Ser26]Aβ 25–40-like antigens were accumulated in the NFT-containing neurons.

To check possible cross-reactivity of 22.7 to intracellular AD-related proteins such as APP, tau, and hyperphosphorylated tau, we performed Western blotting of AD (AD-9 and AD-11) and control (C-5 and C-6) hippocampal homogenates. 22.7 did not label any bands in all AD and control homogenates, whereas antibodies 22C11, tau-1 and AT-8 clearly detected APP, tau, and hyperphosphorylated tau, respectively, in AD homogenates (data not shown). Note that [D-Ser26]Aβ25–40 peptides are undetectable on the Western blot due to their small molecular weights. Antiserum 20.1 showed no cross-reactivity with APP, tau, or hyperphosphorylated tau in both AD and control homogenates (data not shown). Although 20.1 inconsistently, showed 1 weak band of approximately 35kDa in both AD and control homogenates, the band was of similar intensity in both homogenates and was not inhibited by preincubation of the antibody with [D-Ser26]Aβ25–35, suggesting nonspecificity.

**DISCUSSION**

It is generally assumed that Aβ25–35 fragment is only an experimental peptide and does not naturally exist in the brain. However, 20.1 and 22.7 immunoreactivity strongly suggested the presence of truncated Aβ species containing D-Ser26, [D-Ser26]Aβ25–35 and/or [D-Ser26]Aβ25–40, in all AD brains tested, but not inagematched controls. The 2 racemized truncated peptides were each further distinguished by their differential localization among SPs, including the cores, and NFT-containing neurons in AD brains. The 20.1-positive SPs, presumably including [D-Ser26]Aβ25/26–35 antigens (see below), were heavily concentrated in the amygdala and entorhinal cortex where SPs are deposited early in the course of AD (14, 28, 29). In addition, this Aβ species was also detected with the vascular type of Aβ and extracellular NFTs in AD-affected regions. The 22.7-positive neurons, presumably including [D-Ser26]Aβ25–40 antigens, were mostly detected in AD-affected regions such as hippocampal CA1, subiculum, entorhinal cortex and amygdala, but not in the unaffected regions, including CA3 and dentate granule cells (Fig. 5; Table 2).

**Truncated Aβ Species with D-Ser26 in Senile Plaques in AD**

Our results suggest that the 20.1-positive antigens are [D-Ser26]Aβ25–35- or more probably [D-Ser26]Aβ26–35 peptides rather than [D-Ser26]Aβ25–40, because the immunoreactivity in AD brains was completely inhibited by [D-Ser26]Aβ25–35 (Fig. 2E) or [D-Ser26]Aβ25–35 (not shown) but not by [D-Ser26]Aβ25–40 (not shown). The result that [D-Ser26]Aβ1–40 was converted with brain proteases to [D-Ser26]Aβ25/26–35/40 is consistent with the above idea (22).

Racemization of amino acids is a typical age-dependent process. Localization of 20.1-positive truncated [D-Ser26]Aβ peptides in cores of senile plaques, but not in numerous diffuse plaques and relatively sparse neuritic plaques in the AD patients, supports the idea that Aβ proteins may be further modified during age-dependent plaque maturation from diffuse to neuritic plaques in AD brains (30, 31). These observations are consistent with a previous report that racemization of aspartate residues in Aβ was detected in neuritic plaques in AD brains (32, 33).

Aβ proteins in SPs are reported to consist of various lengths of Aβ fragments (20), which is also the case of the racemized Aβ species in AD. In the present study, all of the 20.1-positive plaques colocalized with those stained with 4G8 whose epitope is within Aβ17–24, while some were not stained with 6E10 whose epitope is within Aβ1–17. The 20.1-positive and 6E10-negative plaques are more likely to be mature plaques containing mainly N-terminally truncated Aβ produced during aging by the extensive action of brain proteases such as aminopeptidases. This assumption is supported by the observation that senile plaques are degraded in an age-dependent manner (34, 35).

**Intraneuronal Racemized Aβ Recognized by 22.7 in AD Brains**

The 22.7-positive intraneuronal antigens were present in all AD brains tested, including the 20.1-negative AD
Colocalization of 22.7-positive [D-Ser$^{26}$]Aβ25–40-like antigens and Aβ40 C-terminals or AT8-positive NFTs. Serial hippocampal CA1 cryosections of an AD brain (AD-11) were treated with antigen retrieval with formic acid, and stained with anti-Aβ40 C-terminal antibody (A), AT8 (B), 22.7 (C), or a combination of 22.7 with anti-Aβ40 C-terminal antibody (D) or with AT8 (E). Note anti-Aβ40 C-terminal antibody staining (red) stained many pyramidal neurons in addition to senile plaques. Colocalization of 22.7 staining (blue) with anti-Aβ40 C-terminal antibody staining (red) was also detected in pyramidal neurons (D). E: Double staining with 22.7 (red) and AT8 (blue), 22.7-positive antigens colocalized with AT8-positive NFTs are clearly observed. Scale bar = 50 μm.

brain (AD-8), and were undetectable in neurons with a significant level of NFT in a control subject (C-2) (Table 1), with 22.7, AD and control brains can be clearly differentiated. These antigens are most likely truncated Aβ with an N-terminus of Gly$^{25}$ rather than D-Ser$^{26}$ and with a C-terminus of Val$^{40}$, because the immunoreactivity was inhibited by [D-Ser$^{26}$]Aβ25–40 but not by [D-Ser$^{26}$]Aβ25–35 or [D-Ser$^{26}$]Aβ26–35 (Fig. 5F). This assumption is also supported by the fact that the 22.7-positive neurons remained unstained with 20.1, which binds to Aβ species with D-Ser$^{26}$ at their N-terminus. We also confirmed the colocalization of 22.7-positive antigens and C-terminal fragments of Aβ40 within neurons by sequential immunostaining of C-terminal Val$^{40}$-specific, anti-Aβ40 (Fig. 6D). However, it should be noted that some of the strong, 22.7-positive, neuronal immunostains (approximately 20% of total 22.7-positive neurons) were not completely inhibited even by the highest concentrations of [D-Ser$^{26}$]Aβ25–40 used (Fig. 5F). These clearly 22.7-positive antigens may be [D-Ser$^{26}$]Aβ25–42 derived from [D-Ser$^{26}$]Aβ1–42 or [D-Ser$^{26}$]Aβ25–40 assembled into unusual conformations due to a highly aggregated state or by formation of complexes with other proteins, including NFT. However, it is unlikely that 22.7 directly stained such proteins because 22.7 did not bind AD-related intracellular proteins such as APP, tau, and hyperphosphorylated tau in AD or normal hippocampal homogenates, as assessed by Western blotting.

Several investigators previously reported Aβ-immunoreactivity within neurons and with extracellular NFTs in AD brains (36–39). It has been suggested that the Aβ-immunoreactivity of extracellular NFTs might result from secondary deposition of Aβ onto extracellular NFTs after neuronal death (27). However, the 22.7-positive neurons exhibit similar morphology as unaffected neurons, suggesting that accumulation of toxic [D-Ser$^{26}$]Aβ25–40 may begin early in the course of AD changes, when the neurons may still be intact and functional (Fig. 5A, B, D). This is consistent with recent observation that intraneuronal accumulation of Aβ x-42 was detected in the early stage of AD pathology (40). Of note, however, is that 22.7-positive neurons already contain AT-8-positive hyperphosphorylated tau (Fig. 6E), suggesting an early sign of AD pathology in those neurons.
These results extend our previous observation (8, 22) that toxic Aβ alone, including [D-Ser²⁶]Aβ₂₅–₃₅/₄₀, is not sufficient to produce severe damage of rat hippocampal neurons in vivo but can result in the severe neuronal loss induced by small amounts of excitatory amino acids by enhancing the susceptibility to excitotoxicity. Intraneuronal accumulation of protease-resistant [D-Ser²⁶]Aβ₂₅–₄₀ may further increase neuronal toxicity by rendering neurons susceptible to glutamate possibly released by a hypoxia-ischemic insult in AD, although direct toxicity of the fragments alone can not be excluded.

Serine Racemization Hypothesis in the Pathogenesis of AD: Accumulation, Age-Dependent Racemization at Ser²⁶ Residue, Release, and Processing of β-Amyloids

We have previously shown that [D-Ser²⁶]Aβ₁–₄₀ is soluble and nontoxic but is converted to a toxic fragment such as [D-Ser²⁶]Aβ₂₅–₃₅/₄₀ by brain proteases in vitro and in vivo (22, 23). Based on our present results obtained using AD brains, we have further extended our hypothesis of the involvement of racemization and processing of Aβ in AD neurodegeneration as follows (Fig. 7). 1) Insoluble Aβ₁(Aβ₁–₄₀/₄₂) begins to accumulate in SPs in regions, including the amygdala and entorhinal cortex in the early preclinical stage of AD. 2) Racemization of Ser and Asp residues occurs in an age-dependent manner over the following 20 to 30 years. 3) Aβ₁–₄₀ racemized at Ser²⁶ residue is locally released from the SPs due to its high solubility. 4) The [D-Ser²⁶]Aβ₁–₄₀ and its related peptides are then distributed widely into other areas, including the hippocampus, where they are degraded by brain proteases such as chymotrypsin-like enzymes and aminopeptidases, forming smaller, protease-resistant toxic fragments, [D-Ser²⁶]Aβ₂₅–₃₅/₄₀ (22, 23). 5) [D-Ser²⁶]Aβ₂₅–₃₅/₄₀ may be taken up by hippocampal CA1 pyramidal neurons via endocytosis (41, 42) and exert toxic effects on the neurons by enhancing the susceptibility to excitatory amino acids (8, 9, 22), or by disturbing the neuronal function via binding to cytoskeletal components such as tau and tubulin (43). We do not, however, exclude the racemization of insoluble Aβ within neurons.

The 20.1-positive [D-Ser²⁶]Aβ₂₅/₂₆–₃₅ in the cores of SPs might be derived from residual [D-Ser²⁶]Aβ₁–₄₀ in the SPs, which is finally converted into remnant [D-Ser²⁶]Aβ₂₆–₃₅ by extensive digestion by amino acid and carboxyl peptidases during aging. Activated glial cells are a possible source of such proteases. In contrast to Aβ racemized at the Ser²⁶ residue, Aβ₁–₄₀ and its derivatives racemized at Ser⁸, Asp¹, Asp³, or Asp² residue seem to exert their degenerative effects only on neurons adjacent to senile plaques. This is due to their strong tendency to form fibrils or high susceptibility to brain proteases, including chymotrypsin and aminopeptidases (22, 23).

In conclusion, truncated Aβ species racemized at the Ser²⁶ residue are shown to be present in degenerating neurons and senile plaques in AD patients but not in age-matched controls, and this finding might explain the temporal and topographical inconsistency between the appearance of SPs and the neurofibrillary degeneration in AD. Age-dependent production of [D-Ser²⁶]Aβ₂₅–₃₅/₄₀ in the brain may play a key role in the neuronal degeneration and therefore dementia of AD.
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