Short Communication

Presence of Sodium Dodecyl Sulfate-Stable Amyloid β -Protein Dimers in the Hippocampus CA1 Not Exhibiting Neurofibrillary Tangle Formation

Hiromasa Funato,^{*†‡} Miho Enya,^{*}
Masahiro Yoshimura,[§]
Maho Morishima-Kawashima,^{*} and Yasuo Ihara^{*¶}

From the Department of Neuropathology,^{*} Faculty of Medicine, University of Tokyo, the Japan Society for the Promotion of Science,[†] the Department of Neuropathology,[‡] Medical Research Institute, Tokyo Medical and Dental University, and the Tokyo Medical Examiner's Office,[§] Tokyo, and Core Research for Evolutional Science and Technology (CREST),[¶] Japan Science and Technology Corporation, Kawaguchi, Japan

The amyloid cascade hypothesis of Alzheimer's disease postulates that accumulation of amyloid β -protein ($A\beta$) precedes neurofibrillary tangle formation or neuronal loss in the cortex. Although this temporal profile has been proved in the neocortex by silver staining and immunocytochemical methods, CA1 of the hippocampus exhibits a distinct temporal profile during normal aging: the formation of neurofibrillary tangles precedes senile plaque formation. This temporal profile has been further confirmed by two-site enzyme immunoassay (EIA) quantitation of sodium dodecyl sulfate (SDS)-dissociable $A\beta_{42}$; neurofibrillary tangles are already present despite undetectable levels of SDS-dissociable $A\beta_{42}$. However, when the same specimens were subjected to Western blotting, many cases with or without neurofibrillary tangles showed some accumulation of SDS-stable $A\beta$ dimers that cannot be detected by EIA. Thus, the temporal profile prerequisite for the hypothesis is still valid in CA1, and this finding also suggests that SDS-stable $A\beta$ dimers have some significant effects on CA1 pyramidal neurons, which are most vulnerable to neurofibrillary tangle formation. (*Am J Pathol* 1999, 155:23–28)

($A\beta$), the major component of senile plaques (SPs), leads to the formation of neurofibrillary tangles (NFTs) in susceptible neurons, eventually causing their death, through its neurotoxicity.¹ Two major $A\beta$ species are defined by their different carboxyl-terminal lengths: $A\beta_{40}$ terminating at Val-40 and $A\beta_{42}$ terminating at Ala-42. The former accounts for ~90% of the secreted $A\beta$,² but the latter exhibits a much higher aggregation potential than the former *in vitro*³ and is by far the predominant species of SPs.⁴

The hypothesis is supported by several findings: 1) in the brains from Down's syndrome patients of various ages, the earliest lesion in the neocortex is found to be diffuse plaques consisting exclusively of $A\beta_{42}$,⁵ 2) rare forms of familial AD are caused by mutations of β -amyloid precursor protein (APP),¹ and 3) mutations of presenilin 1 and 2 as well as of APP cause enhanced secretion of $A\beta_{42}$.^{1,2} Along this line of investigation, there is now a large collection of reports describing the neurotoxicity of $A\beta$. Its neurotoxicity is observed *in vitro* under well controlled conditions; fibrillar, but not amorphous, $A\beta$ aggregates are believed to exert toxic effects on cultured neurons.^{6,7} These toxic effects may be mediated through oxidative stress,⁸ although other routes of mediation are also possible.

However, there are some important, but often neglected, observations refuting *in vivo* neurotoxicity of $A\beta$. The most remarkable is that in the hippocampus CA1 and entorhinal cortex, a great number of NFTs often occur without SPs during normal aging^{9–12} and presumably in the initial stage of AD,¹³ as has been repeatedly confirmed by several groups using silver staining or immunocytochemistry. In addition, two lines of transgenic mice exhibiting $A\beta$ deposits did not show NFT formation or neuronal loss, suggesting that $A\beta$ accumulation detected as extracellular $A\beta$ deposits is not sufficient for NFT formation or neuronal loss.^{14,15} These findings led us to

Among the hypotheses that have been proposed thus far on the pathogenesis of Alzheimer's disease (AD), the most dominant is the so-called amyloid cascade hypothesis, which states that accumulation of amyloid β -protein

Accepted for publication March 20, 1999.

Address reprint requests to Dr. Yasuo Ihara, Department of Neuropathology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033. E-mail: yihara@m.u-tokyo.ac.jp.

believe that the amyloid cascade hypothesis of AD is not valid in CA1 and thus may not be so in the neocortex. However, neuropathological findings alone cannot invalidate the amyloid cascade hypothesis, because one cannot exclude the possibility that A β can accumulate to significant levels in CA1 without forming immunocytochemically detectable SPs, and that this invisible but significant accumulation could lead to NFT formation in a subset of neurons and/or their loss in CA1.

In this study, we sought to examine 1) whether NFTs appear in CA1/T4 (medial occipitotemporal cortex) without the accumulation of SDS-dissociable A β 42 as quantitated by the sensitive two-site enzyme immunoassay (EIA)¹⁶ and 2) whether there is a similar temporal relationship of NFT with SDS-stable A β dimers that are detected by Western blotting but not EIA, using specimens obtained from consecutive autopsy cases with ages ranging from 24 to 92 years.¹⁷

Materials and Methods

Subjects

The present study is based on autopsies performed ($n = 74$; 56 men and 18 women) at the Tokyo Medical Examiner's Office (Otsuka, Tokyo).¹⁷ The ages at death of the 74 subjects ranged from 24 to 92 years (3 at 20 to 29 years of age, 4 at 30 to 39 years of age, 17 at 40 to 49 years of age, 18 at 50 to 59 years of age, 13 at 60 to 69 years of age, 10 at 70 to 79 years of age, 8 at 80 to 89 years of age, and 1 at 92 years of age). Postmortem delay ranged from 2 to 24 hours. AD was diagnosed based on both clinical and neuropathological criteria.^{18,19}

Tissue Preparation and Extraction

Cortical pieces, ~80 to 110 mg each, of CA1 and T4 at the level of the lateral geniculate body were sampled from fresh brains at autopsy and stored at -80°C until use. The attached leptomeninges and vessels were carefully dissected out. Cortical blocks from adjacent sites and/or from the same locations on the contralateral side were fixed in 10% buffered formalin and processed for histological and immunocytochemical examination.

Each of the sampled pieces was homogenized as described elsewhere.¹⁷ Five microliters from each homogenate was smeared on polylysine-coated glass slides for tau immunostaining. The remaining part of each homogenate was further homogenized and centrifuged at $100,000 \times g$ for 15 minutes. The supernatant was used for quantitation of A β (soluble A β); the pellet (insoluble fraction) was homogenized in 70% formic acid and the resultant suspension was centrifuged as described above. The supernatant was neutralized with NaOH and trizma base and subjected to the EIA (insoluble A β).²⁰

Enzyme Immunoassay

The two-site EIA for A β consisted of A β monoclonal antibodies BNT77, BA27, and BC05. BNT77 was used as

the capture antibody, and BA27 and BC05 were used as the detector antibodies.²⁰ BNT77, the epitope of which is thought to be located in A β 11-16, is considered to capture all A β species truncated up to position 10, but not p3, which starts at A β 17.²⁰ The BNT77-based EIA employed in this study detects only A β species that run as monomers on SDS-polyacrylamide gel electrophoresis.¹⁶

Western Blotting

Each aliquot of the formic acid extract of insoluble fraction derived from 2.5 mg of tissue was dried by Speed Vac (Savant Instruments, Framingdale, NY) and solubilized with the SDS sample buffer (50 mmol/L Tris HCl (pH 6.8), 12% glycerol, 2% SDS, 2.5% mercaptoethanol, 4 mol/L urea). These samples were subjected to Tris/tricine gel electrophoresis, and the separated proteins were blotted onto a nitrocellulose membrane (pore size 0.22 μm ; Schleicher & Schuell, Dassel, Germany). The blot, after heat treatment,²¹ was incubated with appropriately diluted BA27 or BC05. After washing with Tris/saline-based buffer, the blot was further incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG (Transduction Laboratories, Lexington, KY). Bound antibodies were visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Little Chalfont, UK). This modified version of Western blotting reproducibly detected as little as 20 pg (5 fmol) of A β 1-42 or A β 1-40 per lane, which is equal to 2 pmol/g wet weight.

Besides specimens, synthetic A β 1-40 or 1-42 (10, 20, 50, and 100 pg; Bachem, Torrance, CA) was also loaded onto each gel for Western blot quantitation of A β . SDS-stable A β dimers were quantitated using a standard curve for synthetic A β 40 or 42 (SDS-dissociable A β 40 or 42), and the concentration was expressed as the A β 40 or 42 monomer equivalent. Thus, it was postulated that the blotting efficiency and reactivity of SDS-stable dimers with BA27 or BC05 are the same as those of SDS-dissociable A β 40 or 42.¹⁶ It should be also noted that A β oligomers other than A β dimers are not taken into account.

Semiquantitative Immunocytochemistry for the Abundance of NFTs

The formalin-fixed, paraffin-embedded tissue blocks were cut into 6- μm -thick sections. These sections and smeared aliquots from the brain homogenates were immunostained with anti-human tau by the avidin-biotin method (Vectastain Elite, Vector Laboratories, Burlingame, CA). The abundance of NFTs was assessed by manually counting them on anti-human tau-immunostained sections in five unselected fields under $\times 200$ magnification, and the numbers were averaged. The abundance was rated as follows: -, none; +, 1 to 5; ++, 6 to 10; +++, more than 10 per one $\times 200$ field. The NFT abundance estimated on smears excellently agreed with that obtained from immunostained tissue sections.

Figure 1. The EIA level of insoluble $A\beta 42$ (corresponding to $A\beta 42$ monomers on the Western blot) in CA1 (A) and T4 (C) versus age and the abundance of NFTs in CA1 (B) and T4 (D), based on the consecutive autopsies (age range at death, 24 to 92 years). The detection limit for insoluble $A\beta 42$ (---) by EIA was 12 pmol/g wet weight. Among a total of 74 cases, 40 cases did not show NFTs ($\diamond$) and 29 cases did ($\blacklozenge$) in CA1 (A); and 63 cases did not show NFTs ($\diamond$) and 6 cases did ($\blacklozenge$) in T4 (C), and 5 were AD cases ($\bullet$). $\circ$, non-AD cases in B and D. $P < 0.05$ for indicated comparisons in B and D.

Apolipoprotein E Genotyping

Typing of the apolipoprotein E (ApoE) genotype was performed using the polymerase chain reaction (PCR) as described previously.¹⁷

Results

NFTs Appear without Accumulation of SDS-Dissociable $A\beta 42$ in CA1

In the present series, there were 27 cases showing NFTs but no SPs in CA1, as judged by immunocytochemistry, a result that is in good agreement with the data reported by other groups.^{9–13} In contrast, there was no case showing SPs but not NFTs in CA1. We examined whether there was a temporal relationship between the appearance of NFTs and the accumulation of SDS-dissociable $A\beta$ that is quantitated by BNT77-based EIA.¹⁶ Consistent with the immunocytochemical observations, NFTs occurred without accumulation of SDS-dissociable $A\beta 42$ in the insoluble fraction of CA1 homogenates in 22 cases (Figure 1, A and B). Because EIA is approximately 30-fold more sensitive than immunocytochemical detection of SPs,¹⁷ it seemed that the EIA quantitation may have further confirmed the immunocytochemical data, that the formation of NFTs precedes that of SPs in CA1.

Among the non-AD cases (69 cases), the abundance of NFTs was not correlated with the extent of $A\beta 42$ accumulation (Figure 1B; Mann-Whitney's U test, not significant). Likewise, the appearance of NFTs was independent of the levels of SDS-dissociable $A\beta 42$ in the soluble fraction or those of SDS-dissociable $A\beta 40$ in the soluble

and insoluble fractions of CA1 homogenates (data not shown). However, the formation of NFTs appeared to be related to the age at death of the subjects: none of the patients (24 cases) who died under the age of 50 years exhibited NFTs in CA1. For ages of 50 to 64 years and the age of 65 years and above, the incidence of NFTs increased to 9 of 22 (41%) and 25 of 28 (89%), respectively. NFTs were found in 22 EIA-negative cases in which SDS-dissociable $A\beta 42$ levels were below the detection limit (<12 pmol/g; Figure 1A).

Accumulation of SDS-Dissociable $A\beta 42$ Precedes the Appearance of NFTs in T4

In contrast to CA1, there were only a small number of cases showing NFT formation in T4 (Figure 1, C and D). There were nine cases showing SPs but no NFTs, whereas three cases showed NFTs but no SPs in T4 (data not shown). Twenty-one cases contained detectable levels of SDS-dissociable $A\beta 42$, but no NFTs (Figure 1D). The incidence of NFTs was significantly correlated with the extent of $A\beta 42$ accumulation in T4 (Figure 1D; Mann-Whitney's U test, $P < 0.05$). Furthermore, there was no apparent relationship between the incidence of NFTs and the age of death of the subjects in T4 (Figure 1C).

Presence of SDS-Stable $A\beta$ Dimers in the Presence or Absence of NFTs in CA1

We further assessed by an improved version of Western blotting²¹ the levels of SDS-stable $A\beta$ dimer that cannot be detected by EIA.¹⁶ Representative Western blots of CA1 specimens with BC05, together with BNT77-based EIA values and immunocytochemical findings for SPs and NFTs, are given in Figure 2. Lanes 1, 2, and 3 are CA1 specimens from a 59-year-old man (ApoE genotype: $\epsilon 3/\epsilon 3$), a 61-year-old man ($\epsilon 2/\epsilon 3$), and a 56-year-old man ($\epsilon 3/\epsilon 3$), respectively, in all of whom accumulation of $A\beta 42$ dimers but not SDS-dissociable $A\beta$ was revealed by Western blotting, whereas negligible levels of SDS-dissociable $A\beta 42$ were detected by EIA. Whereas the first two were free from NFTs, the last showed some NFT formation.

Among 32 cases showing negligible levels of SDS-dissociable $A\beta$ by EIA in CA1, 8 showed accumulation of SDS-stable $A\beta$ dimers ($A\beta 42$ dimers, 3 cases; $A\beta 40$ dimers, 1 case; both $A\beta 42$ and $A\beta 40$ dimers, 4 cases) and the presence of NFTs (Figure 3). Eight cases showed accumulation of SDS-stable $A\beta$ dimers ($A\beta 42$ dimers, 4 cases; $A\beta 40$ dimers, 3 cases; both $A\beta 42$ and $A\beta 40$ dimers, 1 case) but no NFTs. Fifteen cases showed neither SDS-stable $A\beta$ dimers nor NFTs. Only one case showed NFT formation without SDS-stable $A\beta$ dimer accumulation. Thus, SDS-stable $A\beta$ dimers accumulated without NFT formation in CA1 (Figure 3, Fisher's exact method, $P < 0.01$), suggesting that SDS-stable $A\beta$ dimers accumulated before NFT formation.

Representative Western blots of T4 specimens are given in Figure 2; lanes 5 and 6 are specimens from a

Figure 2. Representative Western blots of the insoluble fraction of hippocampus CA1 (lanes 1 to 3) and T4 (lanes 5 to 7) homogenates. The formic acid extract (representing A β in the insoluble fraction) was subjected to Western blotting with BC05. Immunocytochemical scores regarding NFTs and SPs, insoluble A β 42 levels quantitated by BNT77-based EIA (pmol/g wet weight), age/gender, and ApoE genotypes are also given. BNT77-quantitated A β corresponds to A β monomers on the Western blot. Lane 4, 50 pg of synthetic A β 1-42. ND, not detected (below the detection limit). The numbers in the left indicate molecular weights of marker proteins in kilodaltons. Open arrow, closed arrow, and arrowheads in the right indicate A β monomer, A β dimers, and putative A β oligomers, respectively.

44-year-old man (ϵ 3/ ϵ 3) and another 44-year-old man (ϵ 3/ ϵ 3), respectively, both of whom exhibited no SPs. One showed no accumulation of A β monomers or A β dimers (lane 5), whereas the other showed accumulation of A β dimers but not A β monomers (lane 6). The third case (66-year-old man; ϵ 3/ ϵ 3) showed significant accumulation of both dissociable A β 42 and SDS-stable A β 42 dimers (lane 7) and SPs but not NFTs.

Among 26 cases showing negligible levels of dissociable A β by EIA in T4, 17 showed accumulation of SDS-stable A β dimers (A β 42 dimers, 4 cases; A β 40 dimers, 9 cases; both A β 42 and A β 40 dimers, 4 cases) but no NFTs (Figure 3).¹⁶ There was no statistically significant difference in the incidence of SDS-stable dimers between CA1 and T4 (χ^2 test). Eight cases showed neither accumulation of SDS-stable A β dimers nor NFTs (Figure 3). Only one case showed both SDS-stable A β dimer accumulation (A β 42 dimers) and NFTs (Figure 3).

Figure 3. The presence or absence of A β dimers as detected by Western blotting versus NFTs in CA1 (left) and T4 (right) among EIA-negative cases.

Thus, SDS-stable A β dimers accumulated without accompanying NFTs in T4, as in CA1.

With regard to the effect of ApoE alleles on NFT formation, 9 of 13 ϵ 3/ ϵ 4 carriers showed NFTs in CA1, whereas 5 of them showed NFTs in T4. Of 58 ϵ 3/ ϵ 3 carriers, 25 showed NFTs in CA1, and 6 of them showed NFTs in T4. There was a higher incidence of NFT in T4 of ϵ 3/ ϵ 4 carriers than ϵ 3/ ϵ 3 carriers (Fisher's exact method, $P < 0.05$, data not shown), but not in CA1 (Fisher's exact method, $P = 0.13$, data not shown).

Discussion

The present study clearly showed that many specimens of CA1 showing NFTs had undetectable levels of SDS-dissociable A β 42. Apparently this result strengthens the view obtained from previous histochemical or immunocytochemical studies⁹⁻¹³ that the amyloid cascade hypothesis is no longer valid in CA1 of the hippocampus. The detection limits of EIA for A β 42 in the soluble and the insoluble fractions are 0.1 pmol/g wet weight and 12 pmol/g wet weight, respectively.^{17,20} The latter value indicates that EIA is approximately 30-fold more sensitive than immunocytochemical detection of A β deposits.¹⁷ From the above, some assumptions (see below) lead us to the following figures by simple calculation: that less than 0.88 nmol/L soluble A β 42 and 105 nmol/L insoluble A β 42 can accumulate in the extracellular space of CA1 showing NFT formation. The A β 42 levels may have been underestimated due to proteolytic degradation during the postmortem period, but this may not be the case with the A β 42 in the insoluble fraction, which appears not to be degraded even up to 18 hours postmortem, in contrast to up to a 50% decrease in A β 42 levels in the soluble fraction during the similar period (Nakabayashi J, Morishima-Kawashima M, Ihara Y, unpublished data). Thus, the A β concentrations in the extracellular space of CA1 showing NFT formation are orders of magnitude lower than those employed for *in vitro* neurotoxicity experiments; neurotoxicity of A β is not observed up to 2.5 μ mol/L in culture medium for dissociated cultured neurons⁸ and 25 μ mol/L for organotypic hippocampal cultures.²² Thus, the CA1 pyramidal perikarya and their dendrites may be bathed in very low, if any, concentrations of soluble or insoluble A β 42. It may be argued that their axon terminals, the most vulnerable portion of the neuron, are located in an area containing higher concentrations of A β , and therefore their neuronal perikarya may suffer the dying-back process.²³ CA1 neurons innervate the subiculum, where subicular neurons in turn project their axons through the fornix to the precommissural areas and mamillary body and to the entorhinal cortex. Although we did not quantitate A β in the subiculum, only one case that showed NFT formation without detectable A β accumulation showed immunocytochemically detectable SPs in the subiculum (data not shown). Obviously, these data alone may have led us to conclude that A β neurotoxicity alone cannot account for NFT formation in CA1.

Because of the undetectability of SDS-stable $A\beta$ dimers by the most commonly used EIA,¹⁶ we undertook the assessment of $A\beta$ monomers (SDS-dissociable $A\beta$) and dimers in these specimens by an improved version of Western blotting. Most unexpectedly, many EIA-negative specimens showed some accumulation of SDS-stable $A\beta$ 40 or $A\beta$ 42 dimers but not of SDS-dissociable $A\beta$ 42.¹⁶ This may suggest that SDS-stable $A\beta$ dimers appear earlier than SDS-dissociable $A\beta$ 42 in CA1 and T4.¹⁶ These SDS-stable $A\beta$ dimers were detected both in CA1 and T4 in approximately one-half of the cases at the age of 40 to 60 years showing negligible $A\beta$ 42 levels by EIA.¹⁶ Thus, it is likely that a much larger amount of $A\beta$ dimers relative to SDS-dissociable $A\beta$ 42 exists in the earliest stage of β -amyloidogenesis.

There were significant differences in the extent of SDS-dissociable $A\beta$ 42 accumulation between CA1 and T4; even when certain cases showed significant accumulation of SDS-dissociable $A\beta$ 42 in T4, they often showed negligible levels of such $A\beta$ 42 in CA1.¹⁷ Thus, it was quite unexpected that the majority of CA1 specimens showing NFTs contained $A\beta$ dimers, and further that many cases without NFTs also contained some $A\beta$ dimers (Figure 3). The incidence of $A\beta$ dimer-positive cases among EIA-negative cases in CA1 is similar to that in T4 (not significantly different, χ^2 test). These results suggest that 1) SDS-stable $A\beta$ dimers may accumulate earlier than NFTs in CA1 (Figures 1 and 3) and 2) such $A\beta$ dimers start to accumulate in CA1 and T4 at a similar time, whereas accumulation of SDS-dissociable $A\beta$ 42 appears to be significantly delayed in CA1 compared with T4.¹⁷ Taken together, the temporal profile of $A\beta$ accumulation and NFTs in CA1 may be as follows: SDS-stable $A\beta$ dimers start to accumulate, followed by NFTs, and SDS-dissociable $A\beta$ accumulation follows, and SPs appear at the end of this sequence. In contrast to CA1, there were many cases showing accumulation of SDS-dissociable $A\beta$ 42 and SPs but no NFTs in T4 (Figure 1). Thus, in T4, SDS-stable $A\beta$ dimers may accumulate first, followed by SDS-dissociable $A\beta$ and SPs. NFTs appear at the end of the sequence. These findings suggest that CA1 may be particularly resistant to the accumulation of SDS-dissociable $A\beta$ 42 for unknown reasons and, furthermore, that SDS-stable $A\beta$ dimers have a distinct metabolic pathway from SDS-dissociable $A\beta$ in CA1.

Given that the mechanism underlying NFT formation during aging is the same in both CA1 and T4, SDS-stable $A\beta$ dimers may be a candidate. More than 2 pmol/g wet weight of SDS-stable $A\beta$ dimers (expressed by $A\beta$ monomer equivalent; see Materials and Methods) may have already accumulated in CA1 showing no NFTs. It is reported that the stratum pyramidale of CA1 exhibits an exceptionally low extracellular volume fraction of 0.12 in rat brain.²⁴ If 1) this can be applied to human CA1, 2) the specific gravity of gray matter is assumed to be 1.0356,²⁵ and 3) SDS-stable $A\beta$ dimers accumulate exclusively in the extracellular space, 17 nmol/L SDS-stable $A\beta$ dimers would accumulate in the extracellular space of CA1 showing no NFTs. Thus, the concentrations of SDS-stable $A\beta$ dimers in CA1 without NFTs are comparable to those employed for *in vitro* neurotoxicity experiments; diffusible,

nonfibrillar $A\beta$ 1-42 oligomers exert neurotoxicity at nanomolar concentrations through an as yet unidentified cell surface receptor and the activation of *fyn*.²⁶ One of the most notable characteristics of SDS-stable $A\beta$ dimers or oligomers is its inability to form fibrils.^{26,27} This presents a sharp contrast to SDS-dissociable $A\beta$ molecules that easily polymerize into fibrils and the amounts are well correlated with amyloid burden.¹⁷ SDS-dissociable $A\beta$ may be essential for SP formation, but not NFT formation.

Currently, we do not know whether the detected SDS-stable $A\beta$ dimers exist in the extracellular space as postulated above or represent the $A\beta$ species in the intracellular compartment. It is possible that these dimers are generated from secreted SDS-dissociable $A\beta$ in the extracellular space.²⁸⁻³⁰ Another possibility is that SDS-stable $A\beta$ dimers accumulate in a certain intracellular compartment of neurons. Consistent with this, SDS-stable $A\beta$ dimers appear to be located in the detergent-insoluble fraction of neuroblastoma cells.³¹ Furthermore, our preliminary data show that the detergent-insoluble, low-density membrane fraction obtained from human brains contains these $A\beta$ dimers. Thus, it is also possible that SDS-stable $A\beta$ dimers detected in EIA-negative specimens represent the $A\beta$ species bound to this particular membrane domain of neural cells.

The origin of the dimers and putative oligomers (see Figure 2) remains to be elucidated. SDS-stable $A\beta$ dimers migrate usually at ~ 6 kd, a little faster than the dimers generated from synthetic $A\beta$ 1-40 or 1-42. A fraction of the dimers is labeled with BAN50 (the epitope in $A\beta$ 1-10), suggesting that their amino termini are not largely deleted. Currently we are not certain as to whether faster migration of the SDS-stable dimers (Figure 2) represent amino-terminal truncation or an aberrant conformation by misfolding, as suggested by others.³⁰ We also do not know whether these dimers and oligomers are covalently linked or noncovalently linked. These molecules are not dissociated into monomers with SDS or other harsh denaturants, including urea and guanidine hydrochloride, raising the possibility of nondisulfide cross-linking. However, these properties do not necessarily indicate that these molecules are covalently linked. For example, the microtubule-binding domain of the tau in paired helical filaments shows similar aggregation that is highly resistant to SDS and guanidine hydrochloride, but appears not to be covalently linked.³² In this case, deamidation and isoaspartate formation in the selected Asn and Asp residues likely have a significant role in SDS-stable oligomerization.³² Possibly, similar post-translational modifications may be at work for the formation of SDS-stable $A\beta$ dimers and oligomers.

One important question remains: which of the $A\beta$ dimers, $A\beta$ 40 or 42, is more important in the initial phase of $A\beta$ dimer deposition? We cannot completely exclude the possibility that $A\beta$ 40 dimers detected in autopsied brains are generated from $A\beta$ 42 dimers by partial proteolysis during the postmortem period. This may be possible because among brains with short postmortem delays there was no $A\beta$ 40 dimer-only brain.¹⁶ However, to confirm this, careful examination of many autopsied cases with short postmortem delays is required. From the pre-

vious data alone, the appearance of A β 42 dimers, but not A β 40 dimers, is age dependent in CA1 and T4.¹⁶ This raises the possibility that A β 42 dimers are the species mainly involved in the deposition, although we cannot exclude the possibility that A β 40 dimers have some important role in the initial stage of A β deposition.

In summary, SDS-stable A β dimers may be a missing link that can resolve an apparent contradiction of the amyloid cascade hypothesis in CA1.

Acknowledgments

We thank Ms. J. Saishoji and Ms. N. Naoi for tissue preparation and Ms. M. Anzai for the manuscript preparation.

References

- Hardy J: Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci* 1997, 20:154–159
- Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S: Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat Med* 1996, 2:864–870
- Jarrett JT, Berger EP, Lansbury PT Jr: The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* 1993, 32:4693–4697
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y: Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A β monoclonals: evidence that an initially deposited species is A β 42(43). *Neuron* 1994, 13:45–53
- Iwatsubo T, Mann DMA, Odaka A, Suzuki N, Ihara Y: Amyloid β -protein (A β) deposition: A β 42(43) precedes A β 40 in Down's syndrome. *Ann Neurol* 1995, 37:294–299
- Pike CJ, Burdick D, Walenciewicz AJ, Glabe CG, Cotman CW: Neurodegeneration induced by β -amyloid peptides *in vitro*: the role of peptide assembly state. *J Neurosci* 1993, 13:1676–1667
- Lorenzo A, Yankner BA: β -Amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. *Proc Natl Acad Sci USA* 1994, 91:12243–12247
- Behl C, Davis JB, Lesley R, Schubert D: Hydrogen peroxide mediates amyloid β protein toxicity. *Cell* 1994, 77:817–827
- Dickson DW, Crystal HA, Mattiace LA, Masur DM, Blau AD, Davies P, Yen SH, Aronson MK: Identification of normal and pathological aging in prospectively studied nondemented elderly humans. *Neurobiol Aging* 1992, 13:179–189
- Braak H, Braak E: Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol* 1991, 82:239–259
- Langui D, Probst A, Ulrich J: Alzheimer's changes in non-demented and demented patients: a statistical approach to their relationships. *Acta Neuropathol* 1995, 89:57–62
- Jellinger KA: Alzheimer's changes in non-demented and demented patients. *Acta Neuropathol* 1995, 89:112–113
- Gómez-Isla T, Price JL, McKeel DW Jr, Morris JC, Growdon JH, Hyman BT: Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci* 1996, 16:4491–4500
- Irizarry MC, Soriano F, McNamara M, Page KJ, Schenk D, Games D, Hyman BT: A β deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. *J Neurosci* 1997, 17:7053–7059
- Irizarry MC, McNamara M, Fedorchak K, Hsiao K, Hyman BT: APP_{sw} transgenic mice develop age-related A β deposits and neuropil abnormalities, but no neuronal loss in CA1. *J Neuropathol Exp Neurol* 1997, 56:965–973
- Enya M, Morishima-Kawashima M, Yoshimura M, Shinkai Y, Kusui K, Khan K, Games D, Schenk D, Sugihara S, Yamaguchi H, Ihara Y: Appearance of sodium dodecyl sulfate-stable amyloid β -protein (A β) dimer in the cortex during aging. *Am J Pathol* 1999, 154:271–279
- Funato H, Yoshimura M, Kusui K, Tamaoka A, Ishikawa K, Ohkoshi N, Namekata K, Okeda R, Ihara Y: Quantitation of amyloid β -protein (A β) in the cortex during aging and in Alzheimer's disease. *Am J Pathol* 1998, 152:1633–1640
- Tierney MC, Fisher H, Lewis AJ: The NINCDS-ADRDA Work Group criteria for the clinical diagnosis of probable Alzheimer's disease: clinicopathological study of 57 cases. *Neurology* 1988, 38:356–364
- Mirra SS, Heyman A, McKee D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L, participating CERAD neuropathologists: The consortium to establish a registry for Alzheimer's disease (CERAD). II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991, 42:1681–1688
- Shinkai Y, Yoshimura M, Morishima-Kawashima M, Ito Y, Shimada H, Yanagisawa K, Ihara Y: Amyloid β -protein deposition in the leptomeninges and cerebral cortex. *Ann Neurol* 1997, 42:899–908
- Ida N, Hartmann T, Pantel J, Schroeder J, Zerfass R, Foerstl H, Sandbrink R, Masters CL, Beyreuther K: Analysis of heterogeneous β A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. *J Biol Chem* 1996, 271:22908–22914
- Bruce AJ, Malfroy B, Baudry M: β -Amyloid toxicity in organotypic hippocampal cultures: protection by EUK-8, a synthetic catalytic free radical scavenger. *Proc Natl Acad Sci USA* 1996, 93:2312–2316
- Vickers JC: A cellular mechanism for the neuronal changes underlying Alzheimer's disease. *Neuroscience* 1997, 78:629–639
- McBain CJ, Traynelis SF, Dingleline R: Regional variation of extracellular space in the hippocampus. *Science* 1990, 249:674–677
- Takagi H, Shapiro K, Marmarou A, Wisoff H: Microgravimetric analysis of human brain tissue: correlation with computerized tomography scanning. *J Neurosurg* 1981, 54:797–801
- Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL: Diffusible, nonfibrillar ligands derived from A β 1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci USA* 1998, 95:6448–6453
- Kuo YM, Webster S, Emmerling MR, De Lima N, Roher AE: Irreversible dimerization/tetramerization and post-translational modifications inhibit proteolytic degradation of A β peptides of Alzheimer's disease. *Biochim Biophys Acta* 1998, 1406:291–298
- Podlisny MB, Ostaszewski BL, Squazzo SL, Koo EH, Rydell RE, Teplow DB, Selkoe DJ: Aggregation of secreted amyloid β -protein into sodium dodecyl sulfate-stable oligomers in cell culture. *J Biol Chem* 1995, 270:9564–9570
- Xia W, Zhang J, Kholodenko D, Citron M, Podlisny MB, Teplow DB, Haass C, Seubert P, Koo EH, Selkoe DJ: Enhanced production and oligomerization of the 42-residue amyloid β -protein by Chinese hamster ovary cells stably expressing mutant presenilins. *J Biol Chem* 1997, 272:7977–7982
- Podlisny MB, Walsh DM, Amarante P, Ostaszewski BL, Stimson ER, Maggio JE, Teplow DB, Selkoe DJ: Oligomerization of endogenous and synthetic amyloid β -protein at nanomolar levels in cell culture and stabilization of monomer by Congo red. *Biochemistry* 1998, 37:3602–3611
- Morishima-Kawashima M, Ihara Y: The presence of amyloid β -protein in the detergent-insoluble membrane compartment of human neuroblastoma cells. *Biochemistry* 1998, 37:15247–15253
- Watanabe A, Takio K, Ihara Y: Deamidation and isoaspartate formation in smeared tau in paired helical filaments: unusual properties of the microtubule-binding domain of tau. *J Biol Chem* 1999, 274:7368–7378