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BIOCHEMICAL EVIDENCE FOR THE LONG-TAIL FORM (Aß1-42/43) OF AMYLOID ß PROTEIN AS A SEED MOLECULE IN CEREBRAL DEPOSITS OF ALZHEIMER'S DISEASE

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SUMMARY We measured the amounts of total Aß, Aß1-40 and Aß1-42/43 in brain tissues using a newly developed ELISA assay and found that the amounts of insoluble Aß1-42/43 and insoluble Aß1-40 were linearly related to the amount of Aß deposits or total insoluble Aß at their lower and higher concentrations, respectively. In an experiment to characterize the Aß species in brain homogenates with buffered saline, we unexpectedly detected soluble Aß which was derived from the insoluble amyloid deposits in brain tissue, indicating reversible depolymerization of Aß from insoluble amyloid deposits. To confirm this finding, we performed 5 consecutive washes of insoluble precipitates of AD brains with buffered saline. Both species of Aß were found in all 5 supernatant fractions and their amounts were gradually decreased. The ratio of Aß1-42/43 to Aß1-40 was increased with the numbers of washes, indicating that Aß1-40 existed in an exposed manner as compared to Aß1-42/43. Thus the present finding is the first biochemical evidence that Aß1-40 was the predominant species involved in the reversible exchanging reaction on seeding Aß1-42/43 between the soluble and the insoluble forms (amyloid fibrils). © 1994 Academic Press, Inc.

Alzheimer's disease (AD) is the most common cause of progressive intellectual failure in aged humans. Characteristic neuropathological changes occur in hippocampus and cerebral cortex as filamentous lesions of cerebral amyloid deposits (senile plaques), in meningeal and cerebral blood vessels (amyloid angiopathy) and of neurofibrillary tangles (1). Because amyloid ß protein (Aß) is the essential constituent protein with a molecular weight of ~4,000 forming senile plaques and amyloid angiopathy (2, 3), it is the hallmark molecule for AD, trisomy 21 (Down's syndrome), hereditary cerebral hemorrhage with amyloidosis (HCHWA)-Dutch type,

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and normal aging. In particular amyloid deposition precedes cognitive symptoms by years or decades, playing a crucial role in the pathogenesis of AD. Molecular biological evidence further indicated the importance of Aß and its precursor protein (APP), the gene of which is located on chromosome 21 (4-7) and was suggested as the causal gene for early-onset familial AD by linkage analyses (8, 9). The production of Aß from APP, though the mechanism of which is not fully understood, is known to be a physiological reaction from the finding of soluble Aß in cerebrospinal fluids (CSF) as well as cell culture media (10, 11). Since amyloid deposition is believed to be a highly specific event in AD etiology, a pathogenic reaction could be speculated to occur leading to conversion of Aß from a soluble form to an insoluble form. There are several possible mechanisms and molecules which might be involved in such pathogenic conversion of Aß including APP mutations (12-15), apolipoprotein E (16), proteoglycan sulfate (17) and radical oxidation (18). Of these factors, the occurrence of the long-tail form of Aß (Aß1-42/43) is particularly interesting in the light of a new hypothesis that Aß1-42/43 plays a role in amyloid seeding and Aß1-40 in elongation of amyloid fibrils on a Aß1-42/43 seed (19). However, little information has been provided on these two Aß species in brain tissues. Here we examined the amounts of each of Aß species with a sensitive sandwich ELISA. We found that the total amount of amyloid deposits is related to the amount of Aß1-40 and that Aß1-40 was relatively more easily removed from amyloid deposits than Aß1-42/43. The present finding may partly account for the previous discrepancy for composition of Aß species and provides a clue to dispute possible roles of the two Aß species from the viewpoint of neuropathology of amyloid deposition.

MATERIALS AND METHODS

Brains.

We used frozen brain tissues which had been taken at autopsy of AD patients and subjects without neurological dysfunction (controls). All the brains were removed within 6 hours of postmortem, and each hemisphere was neuropathologically examined. The other hemispheres were frozen at -80 °C for biochemical studies.

Soluble and insoluble Aß fractions from human brains.

About 1 g wet tissue of the brain cortex was homogenized with 2 volumes of TS buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 0.1 mM DFP, 0.5 mM PMSF, 1 μg/ml TLCK, 1 μg/ml antipain, 0.1 μg/ml pepstatin and 1 μg/ml leupeptin and centrifuged at 100,000 g for 15 min at 4 °C. The supernatant (Sup 1) was carefully separated from the pellets. The pellet was rehomogenized with TS buffer and the resultant supernatant (Sup 2) was recovered. The pellet was extracted 3 more times with TS buffer under the same conditions as described above to obtain Sup 3, 4 and 5.

We also examined insoluble Aß in the SDS-insoluble fraction of brain cortices without purification by RP-HPLC. The insoluble fraction was obtained as follows. Brain cortices were homogenized in 2 % SDS, centrifuged at 100,000 g for 15 min and extracted with 99 % formic acid. The resultant supernatant (10 μl) was neutralized with 1 N NaOH (178 μl) and diluted with 20 mM phosphate-buffered saline (pH 7.0) containing 0.05 % CHAPS. The amounts of Aß1-40, Aß1-42/43 and total Aß in the supernatant were independently quantitated by the sensitive sandwich ELISA using BA-27, BC-05 and BS-85 monoclonal antibodies, respectively.

Sandwich ELISA.

To immunochemically identify and quantitate different species of Aß from brains, we prepared three monoclonal antibodies. One monoclonal antibody (BS-85) against Aß25-35 was used to detect all Aß species in each fraction and the two COOH-terminus-capturing monoclonal antibodies, BA-27 and BC-05, were used to distinguish the two different COOH termini of Aß1-

40 and Aß1-42/43, respectively. The present ELISA assay was established to demonstrate the specificity of these three monoclonal antibodies (described in ref. 20). With these two antibody-dependent sandwich ELISA systems, we confirmed that the monoclonal antibody BA-27 recognized the short-tail form of Aß ending at position 40 and the monoclonal antibody BC-05 exclusively recognized the long-tail form of Aß at position 42/43. The two-site ELISA for Aß was carried out in the same way as described previously. Briefly, 100 μl of standard peptides or samples from fractions was applied onto microplates coated with the anti-Aß (BAN-50) recognizing the NH_2-terminal sequence (1-3) of Aß and incubated at 4 °C for 24 hr. After washing with phosphate-buffered saline, the microplates were incubated with HRP labeled antibodies of BS-85, BA-27 and BC-05 at 4 °C for 24 hr. HRP activities bound to antibody were assayed by color development using the TMB microwell peroxidase system. The recovery rates of insoluble Aß1-40 and Aß1-42/43 were 37 % and 52.6 %, respectively. Those of all soluble Aß species were about 100 %. The reliable assay range was beyond the concentration of 0.16 fmol/well. Inter-/intra-assay coefficient variations were less than 10 %.

RESULTS AND DISCUSSION

Amyloid deposits in brain tissues were found to be composed of heterogeneous species of Aß; the different NH_2-terminal truncation occurred in Aß molecules whose first amino acid started at 1-, 2-, 3-, 4-, up to 11-residues. We also found another modification of pyroglutamate in the NH_2-terminus at 3- and 11-residues of glutamate (22). Despite this heterogeneity, all Aß species were found to have a COOH-terminus of either Val at 40-residue or Ile/Ala at 42/43-residue except for the minor species ending at 39-residue (22). It is interesting to examine the COOH-termini of the Aß species in AD brains because these two Aß species are proposed to have different functions in the formation of amyloid fibrils.

We measured total Aß, Aß1-40 and Aß1-42/43 in amyloid deposits in brains. To examine the insoluble Aß fraction, we extracted the SDS-insoluble fraction from human brain tissues by homogenization with 99 % formic acid without any fractionation such as reversed-phase HPLC, and the extracted samples were then neutralized followed by dilution with buffered saline (Table 1). The recovery rates of insoluble Aß1-40 and Aß1-42/43 were 37 % and 52.6 %, respectively. The low recovery rates, particularly for Aß1-40, occurred during the neutralization from acidic pH to neutral pH. This could be physiologically important by taking into consideration that trans-Golgi network to lysosome where Aß is processsed from APP is in acidic circumstances.

We found that the insoluble amounts of Aß varied from case to case so much. They were 0.38 to 188 nmol/g tissue with a mean of 18.0 nmol/g tissue $\pm$ S.D. of 44.16 for total Aß, and 0.06 to 122 nmol/g tissue with a mean of 12.2 nmol/g tissue $\pm$ S.D. of 29.19 for Aß1-40. In contrast, the amount of Aß1-42/43 was found to show a relatively small variation from 0.027 to 3.42 nmol/g tissue with a mean of 1.00 nmol/g tissue $\pm$ S.D. of 0.98. The amount of total insoluble Aß seemed to be related to the amounts of the two Aß species. To demonstrate this relationship more clearly, the amount of total insoluble Aß was plotted as a function of the amount of insoluble Aß1-42/43 or insoluble Aß1-40 (Fig. 1). We found that the amount of Aß deposits (or total insoluble Aß) was well related to the amounts of insoluble Aß1-42/43 at the lower concentration than ~1 n mol/g tissue (shadow area in Fig. 1a). In contrast, the amount of Aß deposits was linearly related to the amounts of insoluble Aß1-40 at the higher concentration than ~1 n mol/g tissue (shadow area in Fig. 1b). These observations were further confirmed by

Table 1. Amounts of the solubilized and insoluble Aß species

cases	solubilized Aß				insoluble Aß			
	E.I.A.(pmol/g tissue)				E.I.A.(nmol/g tissue)			
	Aß1-42/43	Aß1-40	total Aß	the ratio of Aß1-42/43 to Aß1-40	Aß1-42/43	Aß1-40	total Aß	the ratio of Aß1-42/43 to Aß1-40
AD (case 1)	n.d.	3.30	6.30	-	0.027	n.d.	n.d.	-
AD (case 2)	n.d.	0.90	2.52	-	n.d.	n.d.	n.d.	-
AD (case 3)	1.26	72.0	180	0.018	0.080	2.30	4.25	0.034
AD (case 4)	n.d.	2.19	3.60	-	n.d.	n.d.	n.d.	-
AD (case 5)	0.81	114	156	0.007	0.038	3.51	2.75	0.011
AD (case 6)	0.54	0.63	1.23	0.86	1.18	0.08	1.68	15
AD (case 7)	n.d.	1.38	2.52	-	2.09	0.06	2.50	34
AD (case 8)	0.45	n.d.	1.23	-	0.34	0.16	0.55	2.1
AD (case 9)	1.26	1.86	7.20	0.68	2.57	1.67	5.25	1.5
AD (case 10)	2.31	198	330	0.012	3.42	122	188	0.028
AD (case 11)	1.68	n.d.	13.2	-	1.37	1.46	3.88	0.94
AD (case 12)	2.52	0.90	5.70	0.44	0.30	0.08	0.38	3.6
AD (case 13)	0.81	7.20	11.1	0.11	0.29	0.78	1.55	0.37
AD (case 14)	1.41	23.7	33.0	0.059	0.25	0.92	1.35	0.27
AD (case 15)	1.44	63.0	90.0	0.023	0.99	3.51	6.00	0.28
AD (case 16)	3.90	162	225	0.024	2.47	36.5	46.3	0.068
AD (case 17)	8.40	255	330	0.033	1.06	27.0	31.9	0.039
AD (case 18)	0.90	45.0	60.0	0.020	0.38	4.32	5.00	0.091
AD (case 19)	3.60	0.72	9.00	5.0	0.29	0.08	0.38	3.6
AD (case 20)	12.0	3.90	21.6	3.08	0.91	2.67	4.50	0.34

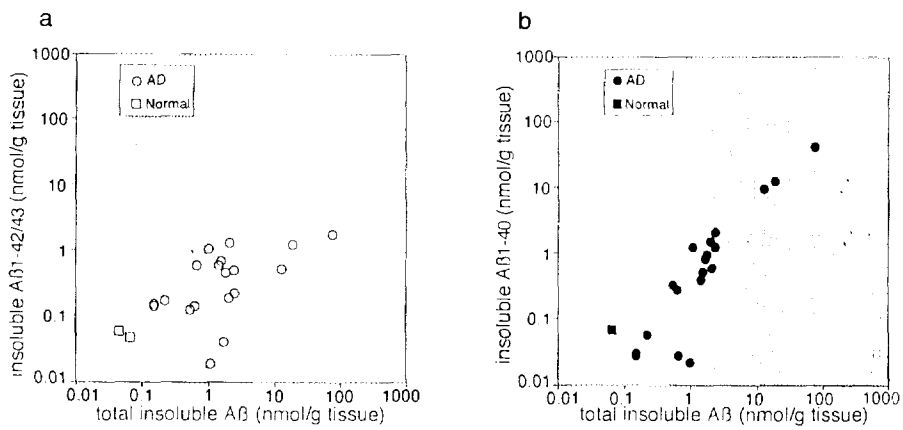

Fig. 1. Relationship among insoluble Aß species of amyloid deposits in cerebral cortices.
The total amount of insoluble Aß was extracted as the soluble fraction with 99 % formic acid, neutralized, diluted and measured with quantitative sandwich ELISA as described in Materials and Methods. The amount of total insoluble Aß was compared with the amount of insoluble Aß1-42/43 or insoluble Aß1-40. Symbols of circle and square represent AD and normal cases, respectively. Open and closed symbols represent Aß1-42/43 and Aß1-40 species, respectively. Symbols in shadow area represent cases with the high correlation (see the text for detail).

calculated correlation coefficient values (r), which were 0.83 for insoluble Aß1-42/43, and 0.99 for insoluble Aß1-40 with lower and higher concentrations of total insoluble Aß, respectively.

These results suggest that Aß1-42/43 is the predominant species of initial amyloid formation and that Aß1-40 is predominantly associated with amyloid deposits in their progressed or late phases. One of these conclusions is in good agreement with the recent hypothesis of Jarrett and Lansbury (19) and the recent observation (23) by immunohistochemistry using the same monoclonal antibodies, BC-05 and BA-27 as we used here. Furthermore, Aß1-40 was proposed to be involved in *in vivo* elongation of amyloid fibrils (19).

While investigating Aß in brain tissues, we found soluble Aß in the supernatant fractions of homogenates of AD brain. However, we supposed that soluble Aß was newly extracted by homogenization in the buffered solution because (1) the amount of total soluble Aß in Sup 1 was in a parallel fashion to the amount of total insoluble Aß, (2) the amounts of soluble Aß in some AD brains were much higher than those in cerebrospinal fluids (~3 pmol/ml) and (3) the amounts of soluble Aß in normal brains were less than 0.05 pmol/g tissue (data not shown). We, of course, could not exclude the possibility that some of the Aß in the supernatant fraction was originally present in the soluble fraction of brain tissue. However, we concluded that the majority of soluble Aß was Aß solubilized *in vitro* (hereafter referred to as solubilized Aß). Although the biological significance of Aß in these fractions might be limited, the amount of total solubilized Aß was found to reflect the amount of total insoluble Aß. The correlation coefficient was 0.71 between insoluble and solubilized Aß. The relationship between the amounts of total solubilized Aß and solubilized Aß1-42/43 was vague as compared to the linear relationship between the amounts of total solubilized Aß and solubilized Aß1-40 (Fig. 2). The correlation coefficient

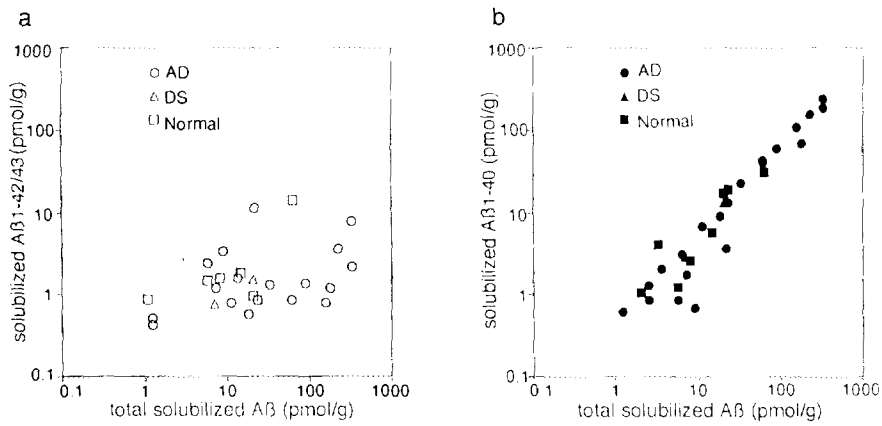

Fig. 2. Relationship among the soluble amounts of total solubilized Aß, Aß1-40 and Aß1-42/43.
Soluble Aß was obtained in the supernatant fraction (Sup 1) after the centrifugation of brain homogenates with TS buffer as described in Materials and Methods. (a) Total solubilized Aß and solubilized Aß1-42/43 and (b) total solubilized Aß and solubilized Aß1-40. Symbols of circle, square and triangle represent AD, normal and Down syndrome cases, respectively. Open and closed symbols represent Aß1-42/43 and Aß1-40 species, respectively.

clearly shows the high value for the latter (r=0.98; closed circles in Fig. 2b) as compared to that for the former (r=0.25; open circles in Fig. 2a).

To investigate the *in vivo* roles of the two species of Aß in brain tissues, we analyzed in detail solubilized Aß and insoluble Aß. As expected, the amounts of solubilized total Aß, Aß1-40 and Aß1-42/43 were smaller than the respective amounts of insoluble Aß species. Moreover, we found another significant difference between solubilized Aß and insoluble Aß. Comparison of Figs. 2a and 2b for solubilized Aß showed clearly that the amount of total Aß was correlated with that of Aß1-40, but was not with that of Aß1-42/43. On the basis of these results, we considered that Aß1-42/43 was not easily solubilized as compared with Aß1-40. In order to further characterize the relationship between Aß1-42/43 and Aß1-40 in brain, we examined the solubility of each Aß species in washing experiments as follows.

Brain tissues were homogenized with TS buffer and centrifuged as described in Materials and Methods. The resultant precipitates were rehomogenized with TS buffer and recentrifuged. These washes were repeated 5 times and the resultant five supernatant fractions were examined to measure the amounts of each Aß species. Figure 3 shows a schematic diagram of the experiment and a graphic summary of the representative results from the brain (case 20); (i) the solubilized Aß was a mixture of two Aß species, (ii) the amounts of total Aß, Aß1-40 and Aß1-42/43 in the supernatants were decreased with increasing number of washes and (iii) the ratio of the amount of Aß1-42/43 to total Aß (or Aß1-40) was increased with increasing number of washes. The present experiment clearly showed that Aß1-40 existed in an easily-washed/solubilized fashion as

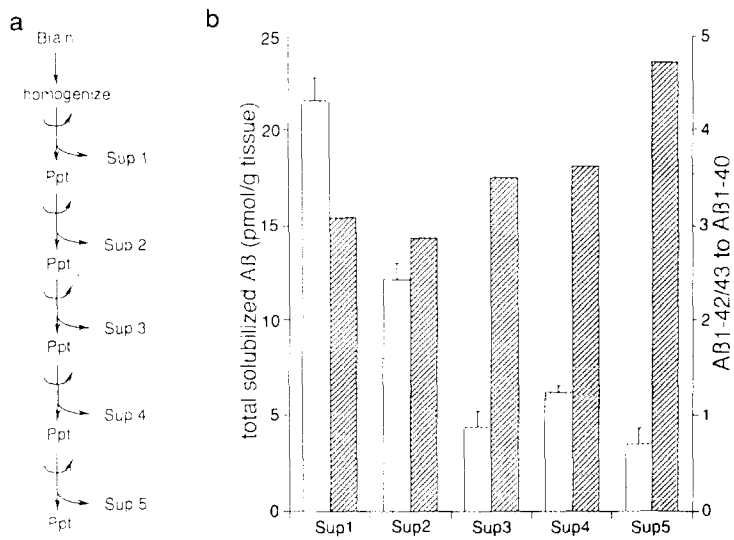

Fig. 3. Differential extraction of Aß1-40 and Aß1-42/43 from cerebral amyloid deposition.
Cerebral cortex tissues (about 1 gram) were homogenized with 2 ml of TS buffer and centrifuged. The resultant supernatant contained the solubilized Aß composed of Aß1-40 and Aß1-42/43. The resultant pellet was rehomogenized with TS buffer followed by centrifugation, producing the subsequent supernatant. (a) A schematic diagram of washing experiment. (b) A graphic summary as for the amount of total Aß (open bar), the ratio (striped bar) of Aß1-42/43 to Aß1-40.

compared with Aß1-42/43. This conclusion was consistent with that from the comparison of solubilized Aß and insoluble Aß (Table 1). We could speculate that insoluble Aß1-40 occurred in an exposed manner as compared to insoluble Aß1-42/43. However, at this moment, we do not know whether this seed of Aß1-42/43 is present in a similar arrangement as seeds in an apple because the morphological entity of the "seed" of Aß1-42/43 before formation of amyloid fibrils is not yet completely clarified. It is probable that the seed of Aß1-42/43 occurs as a diffuse plaque in senile plaques or as an amorphous electron-dense substance.

The present study may have several implications. First, it provides the first *in vivo* biochemical evidence supporting the recent seeding hypothesis (19). Second, it provides evidence of the occurrence of a dissociation reaction from insoluble amyloid deposits to solubilized Aß based on the presence of solubilized Aß in the homogenate supernatants (Fig. 3) in addition to the association reaction demonstrated previously using the paraffin-fixed sections (24). Taken together, these data suggest that amyloid fibrils are formed in a reversible manner. This promptly leads us to another exciting conclusion: amyloid deposition in brains might be halted or reversed through the shift of deposition equilibrium toward the dissociation by the possible drug-treatment. Third, some differences in molecular components of vascular amyloid in previous reports (25-27) may be explained by the present results. The relative amounts of the Aß species in plaque cores, vascular amyloid deposits and diffuse plaques can vary with the degree of amyloid deposition and with the purification procedure as claimed before (22). Aß in an early phase of deposition is predominantly Aß1-42/43 species and Aß in brains with massive amyloid deposition is predominantly Aß1-40 species (Figs. 1a and 1b). As shown in the washing experiment of Fig. 3, the ratio of Aß1-42/43 to Aß1-40 is increased by the washing cycles, indicating that purified insoluble Aß shows relatively lower abundance of Aß1-40 as compared with *in situ* Aß deposits in brain tissue. Thus, it is very likely that Aß undergoes an alteration in composition during isolating operation. Furthermore, the amount of composition of Aß is influenced by other factors such as clinical progression, deposition sources such as amyloid cores, vascular and leptomeningeal Aß deposits (25-27) and genetic background (28, 29). The APP717 missense point mutation (12-15) found in pedigrees of familial AD was recently found to be one of the causes to alter the balance of Aß1-42/43 and Aß1-40 production (20). Aß1-42 was relatively up-regulated in the familial AD brains with APP717 (Val to Ile) point mutation (Tamaoka, in submission) and in cells transfected with the three mutated APP cDNAs at APP717 (Val to Ile, Gly, Phe) that may well account for the reason of early onset of the disease. Apolipoprotein E (Apo E) was reported to be related with late-onset familial AD and to be a risk factor for AD (16). In particular, Apo E allele 4 was shown to enhance amyloid deposition in brains through an unknown molecular mechanism (30). All of the above factors may be involved in the physiological regulation of the concentration of each soluble Aß species in CSF and in the formation of Aß deposits.

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