**β-Amyloid Is Different in Normal Aging and in Alzheimer Disease***

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The mechanism of neurodegeneration caused by β-amyloid in Alzheimer disease is controversial. Neuronal toxicity is exerted mostly by various species of soluble β-amyloid oligomers that differ in their N- and C-terminal domains. However, abundant accumulation of β-amyloid also occurs in the brains of cognitively normal elderly people, in the absence of obvious neuronal dysfunction. We postulated that neuronal toxicity depends on the molecular composition, rather than the amount, of the soluble β-amyloid oligomers. Here we show that soluble β-amyloid aggregates that accumulate in Alzheimer disease are different from those of normal aging in regard to the composition as well as the aggregation and toxicity properties.

A series of evidence indicates that progressive cerebral accumulation of β-amyloid (Ab), a proteolytic product of transmembrane protein APP, is the primary pathogenic event of Alzheimer disease (AD) (1). Recent clues indicate that small, soluble Ab aggregates produce more severe synaptic dysfunction and neuronal damage than do Ab polymers (2–5). This behavior is common to all known pathogenic and nonpathogenic amyloidogenic peptides (6, 7). Soluble Ab is detectable early in the cerebral cortex of subjects at risk for AD pathology, several years before the formation and deposition of amyloid fibrils (8). Hence, the analysis of soluble Ab in brain tissue allows the characterization of the toxic form of the peptide.

A strong argument against the amyloid hypothesis is the abundant and constant deposition of Ab in the brains of elderly subjects, in the absence of signs of neuronal degeneration and dementia (9–11). The reasons for the absence of pathogenic effect exerted by Ab in normal aging are unknown. The issue has important therapeutic implications, because the major strategies to prevent and cure AD are focused on halting Ab accumulation (12).

In brains from Alzheimer disease (AD) and Down syndrome patients, three major species of soluble Ab have been identified by mass spectrometry: the full-length form, Ab1–42, which has a relative molecular mass of 4.5 kDa, and two N-terminal peptides truncated at residue 3 (Ab3–42) and residue 11 (Ab11–42) with relative molecular masses of 4.2 and 3.5 kDa, respectively (13, 14). The 4.2- and 3.5-kDa bands are more prominent in familial AD carrying presenilin 1 mutations than in sporadic AD, suggesting that the ratio of soluble Ab species may dictate the toxicity of the aggregates (15).

We predicted that the composition of soluble Ab underlies the different effect exerted by the molecule in AD and in normal aging. To investigate this hypothesis, we studied the composition and properties of aggregation and toxicity as well as the damage produced on artificial membranes of soluble Ab, comparing these areas in sporadic AD and cognitively normal elderly subjects with abundant amyloid plaques in cerebral cortex.

**MATERIALS AND METHODS**

Tissues—We used frozen blocks and formalin-fixed sections of frontal cortex from 14 cases with late onset sporadic AD (mean age at death 80 ± 8 years) (clinical history of disease; pathological diagnosis according to the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) criteria; post-mortem interval 8 h ± 3) provided by the brain bank of Case Western Reserve University, Cleveland, OH, and from 11 cognitively normally aging (NA) elderly subjects (mean age at death 83.3 ± 10 years; post-mortem interval 9.5 h ± 4). The latter subjects had been tested neuropsychologically annually and agreed to be autopsied for research purposes (provided by the Alzheimer’s Disease Research Center, University of Kentucky). Their neuropsychological scores were within the range of normal. In cerebral cortex abundant Ab plaques were present, with absent or scarce neurofibrillary pathology. The amount of Ab plaques, as shown with monoclonal antibody 4G8, was semiquantitatively evaluated in three nonadajacent sections of frontal cortex and was comparable with that of AD cases.

**Immunoblot Analysis**—Soluble Ab was extracted from the water-soluble fraction of frontal cortex with a well established method described in detail previously (8). Briefly, frozen tissues were homogenized in 4 volumes of saline buffer (50 mM Tris, pH 7.6, 5 mM EDTA, 150 mM NaCl) containing protease inhibitors and centrifuged at 100,000 × g for 1 h. Homogenization was also carried out with EDTA-free buffer. Soluble Ab was immunoprecipitated from the supernatants (1 ml corresponding to 250 mg of tissue) adjusted to 1 × radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% cholic acid,
Characterization of Aβ species in water-soluble fractions of cerebral cortex from NA and AD subjects. A, on immunoblots of immunoprecipitated peptides, the antibody 4G8 detected three bands of 4.5 kDa (B1), 4.2 kDa (B2), and 3.5 kDa (B3) (Fig. 2E). The last two cyclized to pyroglutamate at their N termini (14). Antibodies specific for N-terminal residues 1 and 3 pyroglutamate (α-py3, and α-py11) recognize full-length Aβ (black), and B3 (gray) reveals that cerebral water-soluble Aβ is composed predominantly of Aβ1–42 (50%) in NA and Aβpy3–42 (48%) in AD. Data are expressed as mean values ± S.D.; n = 14 for AD and n = 11 for NA. All data are from triplicate experiments.

MALDI-TOF Analysis—Immunoprecipitated synthetic peptides and tissue samples were all treated under the same conditions. Dried agarose beads were resuspended in 50 μl of 10% formic acid and agitated for 3 h at room temperature. 1 μl of the supernatant was loaded directly onto the MALDI target using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid as matrix. Alternatively, 20 μl of the supernatant was subjected to a single desalting/concentration step before mass spectrometric analysis over a μZipTipC18 (Millipore Corp., Bedford, MA) and eluted in 1 μl of 50% CH3CN + 50% trifluoroacetic acid 0.2%. MALDI-TOF mass measurements were performed on a Voyager-DE STR (Applied Biosystems, Framingham, MA) operated in the reflectron mode. Spectra were calibrated externally using a standard peptide mixture.

Immunocytochemistry—Immunocytochemistry was performed on formalin-fixed, paraffin-embedded sections of frontal cortex. Adjacent 6-μm-thick sections were processed according to the biotin-avidin method using antibodies specific for N-terminal residues 1 and 3 pyroglutamate (14). Sections were pretreated with 98% formic acid for 10 min at room temperature. The reaction was developed with 3,3′-diaminobenzidine as co-substrate. The number of reactive Aβ plaques was determined in 12 fields of the cortex spanning the entire cortical thick-
ness in three AD and three NA cases using an ocular grid of 0.135 mm² at a final magnification of ×100.

Preparation of Peptides—Synthetic peptides corresponding to the three major soluble Aβ species detected by immunoblotting (Aβ1–42, py3–42, and py11–42; Anaspec) were dissolved with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma) to produce a uniform, nonaggregated field of Aβ (12). The studies on toxicity and aggregation are all based on different lots of peptides to avoid the possibility that results could depend on a variation in the synthetic peptide batches (Aβ1–42 lots 26048 and 31475, Aβpy3–42 lots 25479 and 28058, Aβpy11–42 lot 28001; Anaspec). The three synthetic Aβ peptides were suspended in phosphate-buffered saline at a ratio corresponding to the composition of soluble Aβ detected in AD (Aβ1–42, 36%; Aβpy3–42, 48%; Aβpy11–42, 16%) and NA (Aβ1–42, 50%; Aβpy3–42, 29%; Aβpy11–42, 21%) and kept for 24–48 h at room temperature at a final concentration of 10 μM, pH 7.6, for subsequent analyses. For experiments in immunoprecipitation and immunoblotting, peptides were kept at 37 °C for 24 h.

Atomic Force Microscopy—Aggregation was initiated by incubation of the Aβ mixtures corresponding to AD and NA patterns at a concentration of 10 μM in distilled water at room temperature. For AFM analysis, 20 μl-aliquots of the sample were withdrawn at various times during the aggregation reaction, deposited on freshly cleaved mica, and dried under mild vacuum for 30 min. AFM images (amplitude data) were acquired in tapping mode using a Dimension 3000 microscope (Digital Instruments) equipped with a “G” scanning head (maximum scan size 100 μm). Single beam, uncoated, silicon cantilevers were used (type TESP, Nanosensors, and RTESP, Nanodevices). The aggregate sizes were obtained by measuring the aggregate height in cross-section and the corresponding height in tapping mode AFM images.

Thioflavin T Binding Assay—One hundred μl of AD and NA mixtures, 10 μM, were diluted to 1 μM in water, and thioflavin T 6 μM was added to the solution. Fluorescence was measured using a PerkinElmer LS-5 luminescence spectrometer with excitation and emission at 440 nm and 480 nm, respectively, with 5 nm bandwidth. Each sample and standard were done in duplicate.

Membrane Permeability Assay—Vesicle leakage induced by Aβ peptides was evaluated by means of the release of calcein (Sigma) as described previously (16). Aβ aggregates, obtained at different incubation times, were added at a final concentration of 1.5 μM to a phospholipid vesicles suspension (lipid concentration 0.05 mM). To help protein insertion in the membrane, the sample was bath-sonicated for 15 min. The protein aggregation conditions were the same as those used for
MTT reagent was reconstituted in phosphate-buffered saline to 5 mg/ml as described by the manufacturer’s protocol (Sigma). The solubilization solution was 10% SDS in 0.01 M HCl. 10 μl of MTT was added to each well and incubated for 3 h at 37 °C. 100 μl of solubilization solution was added to each well and incubated overnight at 37 °C. The absorbance of the samples was measured at 595 nm (Microplate reader, Bio-Rad).

Statistical Analysis—For statistical analysis, an unpaired Student’s t test with Bonferroni’s post-hoc test was used.

RESULTS

Composition of Soluble Aβ Immunoblot Analysis—We examined by immunoblotting the soluble Aβ species present in the cerebral cortex from subjects with sporadic AD (n = 14) and from cognitively NA-matched controls with abundant amyloid plaques and scarce neurofibrillary pathology (n = 11). As expected (8, 13, 17), following immunoprecipitation with RGP9 antisera and detection with monoclonal antibody 4G8, soluble Aβ resolved into three bands of 4.5 kDa (B1), 4.2 kDa (B2), and 3.5 kDa (B3) in all cases (Fig. 1A). B1 was identified as the full-length Aβ (B1) using an antibody specific for Aβ starting at position 1. The other two bands reacted with antibodies specific for Aβ pyroglutamylated at position 3 (B2) and Aβ pyroglutamylated at position 11 (B3) (Fig. 1B), indicating that B2 and B3 are mostly (see mass spectrometry) composed of modified N-terminal truncated species, as reported previously (13, 15). The antibody specific for Aβ42 recognized all three bands in NA and AD cases, whereas only B1 was labeled by the antibody specific for Aβ40 (Fig. 1C). The intensity of Aβ40 reactivity was equal in NA and AD cases. This experiment indicated that most soluble Aβ belongs to the Aβ42 form and that the Aβ40 species is represented only by the full-length peptide (see mass spectrometry), which likely derives from the parenchymal amyloid angiopathy (18, 19) present in both NA and AD cases.

An identical pattern of Aβ species was observed in immunoblots of brain-soluble fractions either immunoprecipitated with monoclonal antibody 4G8 or analyzed directly following protein precipitation with methanol (Fig. 1D). The results were not influenced by the presence or not of EDTA in the homogenizing buffer.

In its original state, soluble Aβ was present as small aggregates of all Aβ species, which partially disaggregated under reducing conditions of PAGE. This fact was demonstrated by two experiments: (a) the absence of Aβ in soluble fractions filtered through 10-kDa cut-off membranes (Fig. 2C); (b) the co-precipitation of N-terminal truncated Aβ species together with the full-length forms (Fig. 1A) upon immunoprecipitation with the antisera RGP9, which recognized only the first three Aβ residues (Fig. 2A). The state of aggregation of soluble Aβ was confirmed by the presence of several SDS-insoluble oligomers ranging from 6 to 10.6 kDa (Fig. 3). The variable reactivity of the Aβ oligomers with antibodies specific for different Aβ regions likely depends on changes of the epitopes exposure of Aβ species within the aggregates. All of the described bands did not appear when membranes were incubated with the preimmune serum (polyclonal antibodies α-N1 and α-py3). A non-specific reactivity was instead observed above 11 kDa.

When reactivity with the monoclonal antibody 4G8 was considered, B2 was significantly more prominent (accounting for 48% of total soluble Aβ) in AD than in NA brains (p < 0.001), whereas B1 prevailed in NA cases where it represented 50% of the total (p < 0.001) (Fig. 1E). The overrepresentation of B2 or of B1 was observed in each AD and NA case, respectively. An identical ratio among the three bands was observed following detection with the antibodies specific for N termini 1, py3, and py11, indicating that B1, B2, and B3 correspond to the three above

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mentioned peptides. Moreover, when Aβ1–42, Aβpy3–42, and Aβpy11–42 synthetic peptides were mixed in equal amounts and analyzed by immunoblotting with monoclonal antibody 4G8, we observed a reactivity that corresponded perfectly to the relative percentage of each peptide (Fig. 2B). The Aβ1-x/ÅBpy3-x ratio was apparently maintained also in the amyloid deposits in the frontal cortex of AD and NA subjects. In NA cases, the antibody to Aβ1 recognized 3.3-fold more Aβ plaques than the antibody to Aβpy3 (mean number of Aβ plaques in 12 fields of the frontal cortex. Aβ1, 185 ± 42; Aβpy3 56 ± 16) (Fig. 4, A and B), whereas the opposite ratio was observed in AD cases (Aβ1, 75 ± 12; Aβpy3 205 ± 38) (Fig. 4, C and D).

**Mass Spectrometry—MALDI-TOF** mass spectrometry confirmed the presence of Aβ1–42, Aβpy3–42, and Aβpy11–42 as major peptides, as well as the presence of Aβ1–40 and other N-terminal truncated peptides ending at residue 42 (Aβ2–42; Aβ3–42; Aβ4–42) (Fig. 5, A and B). We speculate that the latter truncated Aβ species migrate with B2, the 4.2-kDa band, even if we are not able to confirm it without antibodies specific for the various N termini. However, neither Aβ1–40 nor the non-pyroglutamylated N-terminal truncated species were consistently detectable in all of the AD and NA cases examined (Fig. 5, A and B). The ratio among different Aβ species observed by Western blotting was not evident by MALDI-TOF analysis, where the signals of Aβpy3–42 and Aβpy11–42 are significantly lower than that expected. This is due to a specific suppression of the signal derived from the N-truncated Aβ species, as shown in Fig. 5C and as demonstrated previously with other peptides (20).

**Aggregation of Soluble Aβ**—We examined whether the different representations of soluble Aβ species resulted in different Aβ aggregates. We applied the aggregation protocol that favors the formation of Aβ stable oligomers with a low concentration of short protofibrils (21), because oligomerization is required for Aβ neurotoxicity (2, 5). A mix of the three synthetic Aβ peptides in the ratios corresponding to those detected in NA and in AD were aged at room temperature for 24–48 h at a concentration of 10 µM, and the state of aggregation was observed by AFM. At 24 h, Aβ peptides formed globular structures of 6.4 ± 0.3 nm in diameter, the earliest detectable form of Aβ aggregation (22, 23). Although these aggregates were present in both mixtures, they were clearly more numerous in the AD mixture (Fig. 6A). After 48 h of incubation the AD mixture displayed 12-nm-thick protofibrils, resulting from the assembly of thin (3–4 nm) subunits, which were absent in the NA mixture (Fig. 6B).

To measure the aggregation properties, we analyzed the two pools of synthetic peptides using a thioflavin T fluorimetric assay. The AD mixture revealed a higher rate of aggregation than the NA mixture, and the difference became statistically significant after 48 h (Fig. 6C,*, p < 0.05). This result was further confirmed by sedimentation assay (data not shown).

**Toxicity of Soluble Aβ**—The effect of soluble Aβ pools on neurons viability was assessed by incubating neuroblastoma cells with the two different mixtures of synthetic peptides at 1 µM in culture media. We used as the parameter of cell viability the MTT assay, which has been known to be a sensitive indicator of Aβ-mediated toxicity (22). After 24 h of treatment the AD mixture produced a 30% decrease of cell survival statistically different from that of untreated cells (**, p < 0.01) in comparison with a 20% decrease induced by the NA mixture (non-significative) (Fig. 6D). After 48 h of incubation, the AD and NA mix-
tutes, respectively, determined a 50 and 30% decrease of cell survival, and the difference of the toxic effect of the two types of Aβ aggregates was significant (Fig. 6D, *p < 0.05). The scrambled peptide Aβ42–1 used as a negative control had no effect on cell survival (Fig. 6D).

Effect of Aβ Species on Membrane Permeability—Because cytotoxicity of Aβ oligomeric species has been ascribed to the ability of Aβ to generate membrane pores (24), we analyzed the permeabilization of liposome membranes induced by early aggregates of Aβ1–42 and Aβ1–42, the predominant peptides of soluble Aβ in NA and AD. We measured calcein release from unilamellar vesicles made of neutral and negatively charged phospholipids as an index of membrane permeabilization, as described previously (16). After 12 h of aggregation, Aβ1–42 caused a 23% increase of membrane permeability, which instead was only slightly altered by Aβ1–42 (Fig. 7). The opposite conditions were observed with a longer time of incubation (24 h) of the peptides (Fig. 7), suggesting that Aβpy3–42 more quickly reaches the state of aggregation that produces the highest membrane damage.

DISCUSSION

Our findings show that the soluble Aβ aggregates present in AD consistently and significantly differ in composition from the aggregates associated with NA and exhibit a higher neurotoxicity, which can be correlated with the predominance of the N-terminal truncated species over the full-length form. Among all N-terminal truncated peptides, Aβ3–42 is the prominent form, as we showed by comparing the reactivity of its specific antibody with that obtained with the monoclonal antibody 4G8 that recognizes all Aβ species (Fig. 1B). The prevalence of Aβ3–42 over the other N-terminal truncated species that probably migrate with the 4.2-kDa band (B2) (Aβ2–42, Aβ1–42) is confirmed by mass spectrometry analysis, which consistently revealed only Aβ3–42 in all cases examined. The high relative amount of Aβ3–42 on the total Aβ load was reported previously by Harigaya et al. (25) using a specific sandwich ELISA. The highly pathogenic effect of Aβ3–42 is supported by the finding that the Aβ3–42 early aggregates alter the membrane permeability, suggesting that they form pores in the membrane as it has been proposed for other amyloidogenic peptides. Thus, depending on the relative representation of N-terminal truncated species, Aβ aggregates may be associated with very severe degeneration, as in the case of mutant presenilin 1 (15), or may exert a lower toxic effect, as in NA.

The Aβpy3–42 species might be generated from APP through an alternative β-secretase cleavage or they can be produced from the full-length 1–42 form by extracellular aminopeptidases and modified by glutaminyl cyclase to generate pyroglutamate (27). Aβ-Amyloid-cleaving enzyme 1 (BACE1) is responsible of cleavages at position 1 and 11 of Aβ (28), and Aβ fragments starting with residue 3 were never reported when analysis of APP processing was carried out in vitro (29). However, in a double presenilin 1/APP mutant mice the Aβpy3–42 form was detected (30), suggesting that in vivo BACE1 or another still unknown endoprotease might produce the cleavage at position 3. Following cyclization of N-terminal glutamate, Aβ may acquire partial resistance to most of the extracytoplasmic aminopeptidases (31–33), with ensuing accumulation of Aβpy3–42 in AD brains. Moreover, the proteolytic cleavage of Aβ N-terminal cyclized fragments requires neprilysin (34–36), a specific metallopeptidase that is reduced in AD (37). Therefore, proteolysis of Aβ at its N terminus, by limiting the rate of Aβ catabolism and enhancing its seeding capacity and toxicity, may play a critical role in the pathogenesis of AD. Therapeutic strategies in AD should particularly target Aβ species truncated at the N terminus.

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FIGURE 7. Calcein release induced in 1:1 molar ratio phosphatidylcholine/phosphatidylserine liposome membranes by Aβ aggregates monitored as a function of protein aggregation time. Early Aβpy3–42 (A) and Aβ1–42 (B) aggregates produced after 12 h of incubation cause a 23 and 5% increase, respectively, in membrane permeability. The difference in calcein release induced by the two peptides is less after 24 h of incubation. The data are expressed as a percentage of the maximum fluorescence, determined from the ratio (F − F0)/F0, where F0 is the fluorescence intensity before protein addition, F is the fluorescence measured during the release experiment, and Fmax is the maximum fluorescence, determined by adding 0.5% (w/v) sodium cholate at the end of the experiment to disrupt the vesicles and obtain complete content release.
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