Morphology and Toxicity of Aβ-(1-42) Dimer Derived from Neuritic and Vascular Amyloid Deposits of Alzheimer’s Disease*

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In the course of analyzing the chemical composition of Alzheimer’s disease neuritic and vascular amyloid, we have purified stable dimeric and trimeric components of Aβ peptides. These peptides (molecular mass 9.0 and 13.5 kDa) were separated by size exclusion chromatography in the presence of 80% formic acid or 5 M guanidine thiocyanate, pH 7.4. The average ratio of monomers, dimers, and trimers was 55:30:15, respectively. Similar structures were produced over time upon incubation of synthetic Aβ-(1-42) at pH 7.4. The stability of these oligomeric forms was also demonstrated by Western blot and mass spectrometry. Atomic force microscopy and electron microscopy rotary shadowing revealed that the monomers polymerized into 8-10 nm filaments, whereas the dimers generated prolate ellipsoids measuring 3-4 nm in diameter. The pathogenic effects of the dimeric Aβ-(1-40/42) were tested in cultures of rat hippocampal neuron glia cells. Only in the presence of microglia did the dimer elicit neuronal killing. It is possible that these potentially pathogenic Aβ-(1-40/42) dimers and trimers from Alzheimer’s disease amyloid represent the soluble oligomers of Aβ recently described in Alzheimer’s disease brains (Kuo, Y.-M., Emmerling, M. R., Vigo-Pedfrey, C., Kasunic, T. C., Kirkpatrick, J. B., Murdoch, G. H., Ball, M. J., and Roher, A. E. (1996) J. Biol. Chem., 271, 4077-4081).

Although evidence implicates β-amyloid peptide (Aβ) in the pathogenesis of Alzheimer’s disease (AD)† (reviewed in Ref. 1), little is known about the nature of the Aβ mediating the pathology. Toxicity initially was attributed to aggregated Aβ in amyloid plaques (1), the morphological hallmarks of AD brains. Aβ-(1-42) is the major peptide constituent of amyloid plaques (2), and increased production of the 42-amino acid peptide correlates with an earlier onset of AD (1). However, recent studies show that small quantities of Aβ-(1-42) also exist as soluble peptide in the plasma, cerebrospinal fluid, and cerebral cortex of AD and normal individuals and are also secreted by cells in tissue culture (3-13). Utilizing ultracentrifugation, graded membrane filtration, and ELISA, we have recently isolated and quantitated the oligomeric water-soluble Aβ present in the brains of AD and control individuals (13). The levels of insoluble Aβ in AD brains are at least 100 times higher than those found in control brains. The amounts of water-soluble Aβ in AD brains are approximately six times higher than those detected in control brains. Interestingly, we isolated an Aβ fraction, from the Aβ water-soluble oligomeric pool, with a molecular mass of <10 kDa containing monomeric and/or dimeric forms of Aβ peptide (13). In all probability these peptides represent the initial building blocks that may ultimately aggregate into insoluble Aβ filaments. In the course of analyzing the chemical composition of AD neuritic plaque and vascular amyloid, we have purified stable dimeric and trimeric components of Aβ-(1-40/42) (2, 14–15). In the present study we report the chemical and morphological characteristics of the dimeric Aβ as elucidated by atomic force microscopy and transmission electron microscopy techniques. In addition, the potential for toxicity of the AD brain-derived Aβ-(1-40/42) dimer was assessed on glial-neuronal cell cultures.

MATERIALS AND METHODS

Purification of Aβ-(1-42) from AD Brain—Brains were obtained from eight patients who died of AD (postmortem delay 3–6 h). After separation of the leptomeninges, the right hemispheres were frozen at –70 °C. Examination of the left hemispheres revealed numerous neuritic plaques and neurofibrillary tangles fulfilling the diagnostic criteria of AD as established by the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) (16). The cerebral cortices were minced, gently homogenized, and submitted to a series of isocratic sucrose discontinuous sucrose density gradients were performed as described previously (2). The amyloid cores collected at the 1.4–1.7 M sucrose interface were solubilized in formic acid and, following separation of insoluble lipofuscin by high speed centrifugation, dialyzed against 6 M guanidine HCl (2). The resulting white flocculent precipitate (containing protease inhibitors (2). Collagenase digestion, SDS lysis, and discontinuous sucrose density gradients were performed as described previously (2). The amyloid cores collected at the 1.4–1.7 M sucrose interface were solubilized in formic acid and, following separation of insoluble lipofuscin by high speed centrifugation, dialyzed against 6 M guanidine HCl (2). The resulting white flocculent precipitate (containing protease inhibitors) was recovered by centrifugation, and both pellet and supernatant (predominantly Aβ-(1-40)) were submitted to Superose 12 column (Pharmacia, Upsala, Sweden) chromatography developed with 80% glass distilled formic acid (2). Purification of amyloid peptides from leptomeningeal vessels followed the protocol already published by our laboratory (14). Synthetic peptides (Aβ-(1-40) and -(1-42)) were either...
obtained from Bachem (Torrance, CA) or from California Peptide Research Inc. (Napa, CA). SDS-polyacrylamide gel electrophoresis was carried out on a 10–20% Tris-Tricine gradient gel (Novex, San Diego, CA). Peptides were transferred onto nitrocellulose membranes and reacted with antibody 4G8 (Zenetek, Maryland Heights, MO) against Aβ residues 17–24.

Atomic force microscopy (AFM) and transmission electron microscopy—Aβ specimens were prepared for AFM analysis on a pyrolytic graphite (2YB Grade, Advanced Ceramics, Cleveland, OH). Image data were generated on a NanoScope III MultiMode scanning probe workstation (Digital Instruments, Santa Barbara CA). A full description of the methodology has been previously reported (17). For electron microscopy, platinum replicas were prepared according to previously published methods (18). Mica slabs containing the Aβ samples were rotary-shadowed with platinum in a Barlow’s freeze-fracture apparatus. Platinum replicas were placed on copper grids and examined with a Phillips Electron Microscope 300.

Morphology and Neurotoxicity Assays—Rat microglia were isolated from confluent dissociated brain cell cultures by rotatory shaking for 18 h (19). Cells were placed in N2 media containing gentamicin (48 μg/ml) and recovered by selective adhesion to plastic after 2 h. Highly enriched populations (>95% homogeneity) were identified by scanning EM, presence of phagocytic activity, and presence of scavenger receptor using Ac-LDL bound to the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetraethylindocarbocyanine (DiI-Ac-LDL; BioMedical Technologies Inc.).

Cultured neurons prepared from rat hippocampus were used in toxicity studies as described previously (20). Briefly, rat hippocampal neurons (fetal day 18) were plated onto poly-L-lysine-coated coverslips in 24-well plates at 250,000 cells per well in N2 culture media and 5% fetal bovine serum. Gradual reduction of serum began on day 7 in vitro by 1:1 volume replacement with chemically defined media. Cultures consisted of process-bearing neurons (10–20% of total cell population) on top of a bed of astroglia (70%–90%). In order to eliminate microglia, cultures were exposed to saporin, a ribosome-inactivating protein (21) coupled to acetylated LDL (Ac-LDL), on culture day 8, at concentrations of 1 μg/ml. Saporin-Ac-LDL selectively bound to scavenger receptors and reduced microglial numbers to ~0.1% of the total population but had no effect on the viability of either neurons or astroglia (data not shown). After 14 days in vitro, cultures (with a final concentration of 0.6% serum) were exposed to Aβ peptides for 72 h. Cells were fixed in 3% paraformaldehyde at room temperature for 6 h and immunostained by overnight incubation with a mixture of anti-neurofilament antibodies (SMI-311, 1:150; RT-97, 1:150; Sternberger Monoclonals, Inc.) plus anti-MAP-2 (Boehringer Mannheim; 1:200) at 4°C in the presence of 2% horse serum and 0.3% Triton X-100. Indirect immunofluorescence labeling used biotinylated goat anti-mouse IgG (Vector Laboratories, 1:200) and rhodamine streptavidin (Jackson Immuno-Research, 1:1200) in buffered saline, pH 7.6.

RESULTS AND DISCUSSION

Amyloid proteins purified from AD neuritic plaque cores or from the leptomeningeal vessels can be fractionated into five components by size exclusion chromatography in the presence of either 80% formic acid or 5 M guanidine thiocyanate, pH 7.4. Three of these fractions (molecular mass 4.5, 9.0, and 13.5 kDa) corresponded to monomeric, dimeric, and trimeric Aβ (Fig. 1A).

Of the total neuritic plaque Aβ shown in Fig. 1A, 85–95% represents Aβ-(1–42) and 5–15% represents Aβ-(1–40) (2). In contrast, the Aβ-(1–42) in the leptomeningeal vessels ranges from 40 to 60% (14). The presence of Aβ-(1–42) was demonstrated by tryptic digestion and CNBr cleavage that yielded the 36–42 peptide of Aβ. The quantitative relationship between monomeric, dimeric, and trimeric Aβ-(1–40) and -(1–42) together, recovered from several independent preparations from amyloid cores and vascular amyloid, can be expressed as an average ratio of 55:30:15 (monomer: 48 – 65% dimer: 21 – 36%; trimer: 12 – 21%), respectively. The ratios were determined by integration of the area defined by the chromatographic peaks.

Column chromatography of synthetic Aβ-(1–42), in different concentrations of formic acid (80, 60, 40%), did not change the ratio between monomers and dimers (data not shown). When the concentration of the formic acid was reduced to 20%, most of the peptide aggregated at the top of the chromatographic column. However, the small amount of Aβ-(1–42) eluted at this concentration still demonstrated the presence of both Aβ monomer and dimer.

Synthetic Aβ-(1–40) was freshly dissolved and chromatographed in 80% formic acid. As a percentage of the total Aβ and determined by integration of peak area, dimeric Aβ yielded approximately 1%. However, synthetic peptide Aβ-(1–42), under the same conditions, generated on the average 7.4% (range: 7.0–7.7%, n = 12) dimeric Aβ. This suggests that the ability to make stable dimers, in freshly prepared solutions of synthetic...
Aβ, may depend upon the presence of the two C-terminal residues Ile-41 and Ala-42. Interestingly, re-chromatography of the AD and synthetic dimeric Aβ-(1–42) failed to produce the monomeric fraction. This suggests that the dimer appears to have a very stable conformational association and that monomers and dimers are not in equilibrium under these experimental conditions. However, incubation of the chromatographically purified Aβ-(1–42) monomer at 37 °C, in 20 mM Tris-HCl, pH 7.4, for a period of 12 h to 4 weeks generated increasing quantities of dimer/trimeric Aβ-(1–42) (Fig. 1B). Re-chromatography of the dimeric/trimeric Aβ-(1–42) for various times always failed to yield the monomeric form.

Tryptic digestion and CNBr cleavage of the neuritic plaque and leptomeningeval Aβ-(1–42) dimer yielded the same high performance liquid chromatography peptide maps as those observed for Aβ-(1–42) monomer (2, 14, 15). The stability of the Aβ peptides was also demonstrated by Western blot where the Aβ-(1–42) monomer, dimer, and trimer retained their expected molecular mass (Fig. 1A). Likewise, matrix-assisted laser desorption ionization mass spectrometry of synthetic Aβ-(1–42) confirmed the molecular mass of the monomeric, dimeric, and trimeric forms (Fig. 1C).

Atomic force microscopy (AFM) of the monomeric and dimeric fractions of AD-derived Aβ revealed a contrasting morphology. Upon removal of formic acid, by dialysis against water followed by 20 mM ammonium bicarbonate, the monomeric Aβ-(1–42) polymerized into an array of 8–10-nm diameter filaments (Fig. 2A). By contrast, the dimeric Aβ-(1–42) generated granular particles affecting the shape of prolate ellipsoids measuring 3–4 nm with occasional aggregation into larger conglomerates. The 3–4-nm form may be interpreted as the basic dimeric structure. Larger forms, 7–8 nm or greater, could correspond to oligomeric aggregates of Aβ-(1–42).

Platinum replicas of monomeric and dimeric AD-derived Aβ-(1–42) examined by electron microscopy also showed the monomer polymerized into 10-nm filaments (Fig. 2C). At higher magnification filamentous structures, with an axial periodicity of 20 nm, were clearly discernible in the platinum-coated structures (Fig. 2C, inset). This rope-like morphology may result from the intertwining of two proto-filaments such as those previously observed in re-polymerized Aβ derived from the AD brain (15). The 9-kDa fraction, on the other hand, revealed no filaments but rather globular particles and random aggregates similar to those observed by AFM (Fig. 2D).

The biological effects of the monomeric and dimeric Aβ-(1–42) fractions, obtained from both the neuritic plaques and the leptomeningeval vascular deposits, were investigated by application to astroglial-neuronal cultures. Primary cultures of rat hippocampal neurons demonstrated a robust appearance when plated on top of a bed of astrocytes in the presence of microglial cells (Fig. 3A). The latter cells were then selectively eliminated. Neurons exhibited a dramatic loss of viability in the presence of even small doses (4 μg/ml) of monomeric (data not shown) or dimeric AD-derived Aβ-(1–42) (Fig. 3B), when microglial cells were added back to the cultures. The average percentage of neuronal death, expressed as mean and standard error of the mean from triplicate experiments, was 60 ± 3% and 55 ± 3% for monomeric and dimeric Aβ-(1–42), respectively, compared with Aβ-treated microglia-free sister cultures (Fig. 3C). These latter neurons unexpectedly exhibited no loss of viability when exposed to as much as 40 μg/ml monomeric or dimeric Aβ-(1–42), in the absence of microglial cells. The lack of direct neurotoxicity of Aβ is in agreement with previous reports that show that such an effect is readily demonstrated only in low density or otherwise stressed cultures (22, 23). In our culture system, plated at high cell densities, a stable neuronal network is maintained among a population of supportive astroglia, a situation we believe to more closely approximate conditions found...
for the Aβ-induced microglial toxicity described here, however, is the neurotoxic phenolic amine recently reported to be released by microglial cells after in vitro exposure to isolated AD plaque cores (20). It is not apparent from the present work whether activation of the microglia to become neurotoxic is due to the Aβ monomer itself or to one of its aggregated products. In the case of the dimeric Aβ-(1–42), however, the observed microglia-dependent toxicity must be mediated through the non-polymerized form of the peptide since the Aβ dimers fail to form fibrils. The inability of the dimeric Aβ to make fibrils can be explained by its apparent morphology.

The molecular size of the dimeric Aβ may facilitate the diffusion of such molecules within the intricate narrow extracellular space (10–24 nm) of the cerebral cortex. The possible effects of dimeric Aβ within the AD brain are suggested by our cell culture studies. The ability of the Aβ-(1–40/42) dimers to cause neurotoxicity in the presence of microglia shows that the dimer is possibly yet another agent of Aβ-related pathology. The potential diffusibility of the molecule may also explain the reported discordance between the amount of fibrillar amyloid deposits and the extent of neuronal pathology observed in some AD brains (26, 27). The toxicity of the dimeric Aβ-(1–40/42) also may not be limited to nerve cells. Van Nostrand's laboratory (28) reported that human leptomeningeal smooth muscle cells also exhibit degenerative changes when exposed to the nonaggregated form of Aβ-(1–40/42). These phenomena are not observed when the cells are exposed to the aggregated form of the peptide.

It is still unclear how soluble amyloid is recruited into fibrils and to what extent it plays a role in AD neuropathology. Soluble Aβ has been detected in plaque-free Down’s syndrome brains (12), at levels (20.4 ± 6.5 ng/g) intermediate between that of control and plaque-ridden Down’s syndrome brains. This report did not distinguish between Aβ-(1–40/42) and Aβ-(1–40). Nevertheless, there is an elevation in the pool of soluble amyloid prior to the detection of fibrillar Aβ. Likewise, in a study utilizing the transgenic mouse FVB/N, transfected with the human β-amyloid precursor protein, the reported amount of Aβ-(1–40/42) was 62 ng/g tissue (29). Histopathological analysis of these mice revealed no extracellular deposits of fibrillar amyloid despite the presence of severe behavioral changes, altered brain physiology, extensive gliosis, and premature death. The Aβ present in these transgenic mice may represent monomeric and oligomeric soluble forms of the peptide, and it may be these forms that produce the associated pathology. Similar amounts of water-soluble monomeric/oligomeric Aβ-(1–40/42) have been reported by our group in AD brain where the average amount of Aβ represents approximately 40 ng/g of cerebral cortex (13).

In the present report we demonstrate that Aβ dimer/trimer forms are present in purified neuritic plaque cores and in sheets of vascular amyloid. This finding allows two interpretations. First, stable soluble monomeric and oligomeric forms of Aβ may be readily incorporated into the amyloid fibrils. This proposition is favored by our previous finding that soluble monomeric and oligomeric Aβ can be isolated from the brain by ultracentrifugation and sieving techniques (13). Also, small amounts of SDS-soluble monomeric and oligomeric Aβ (4, 6, 8, and 12 kDa) have been detected in the conditioned media of β-amyloid precursor protein transfected Chinese hamster ovary cells (11), and, using chromatographic techniques, stable dimeric- and trimeric-soluble Aβ peptides have been demonstrated in human cerebrospinal fluid (30). The alternative explanation for our present findings is that oligomeric forms of Aβ may also arise from a result of association of monomers within the filament structure as a function of time. Presently, we

\footnote{D. Giulian, personal communication.}
cannot exclude either of the interpretations of our findings outlined here.

Consistent with our experiments and those of other investigators, we wish to propose a scenario in which the cores and wisps of filamentous amyloid found in the neuritic plaques represent a depository in which toxic soluble monomeric/oligomeric Aβ is concentrated and its associated pathology confined to the sites of deposition. The frequent and intimate association of plaques with capillaries (31), and in particular with the basal lamina of these vessels (14), may be indicative of aborted attempts to rid the neuropil of this substance. The vast network of astrocytes in the cerebral cortex is one cellular candidate to attempt to remove the soluble or aggregated amyloid, may produce further neuronal damage, and a cycle of inflammatory responses in AD brain ensues (36).

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