The Ratio of Monomeric to Aggregated Forms of Aβ40 and Aβ42 Is an Important Determinant of Amyloid-β Aggregation, Fibrillogenesis, and Toxicity

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Aggregation and fibril formation of amyloid-β (Aβ) peptides Aβ40 and Aβ42 are central events in the pathogenesis of Alzheimer disease. Previous studies have established the ratio of Aβ40 to Aβ42 as an important factor in determining the fibrillogenesis, toxicity, and pathological distribution of Aβ. To better understand the molecular basis underlying the pathologic consequences associated with alterations in the ratio of Aβ40 to Aβ42, we probed the concentration- and ratio-dependent interactions between well defined states of the two peptides at different stages of aggregation along the amyloid formation pathway. We report that monomeric Aβ40 alters the kinetic stability, solubility, and morphological properties of Aβ42 aggregates and prevents their conversion into mature fibrils. Aβ40, at approximately equimolar ratios (Aβ40/Aβ42 ~ 0.5–1), inhibits (>50%) fibril formation by monomeric Aβ42, whereas inhibition of protofibrillar Aβ42 fibrillogenesis is achieved at lower, substoichiometric ratios (Aβ40/Aβ42 ~ 0.1). The inhibitory effect of Aβ40 on Aβ42 fibrillogenesis is reversed by the introduction of excess Aβ42 monomer. Additionally, monomeric Aβ42 and Aβ40 are constantly recycled and compete for binding to the ends of protofibrillar and fibrillar Aβ aggregates. Whereas the fibrillogenesis of both monomeric species can be seeded by fibrils composed of either peptide, Aβ42 protofibrils selectively seed the fibrillogenesis of monomeric Aβ42 but not monomeric Aβ40. Finally, we also show that the amyloidogenic propensities of different individual and mixed Aβ species correlates with their relative neuronal toxicities. These findings, which highlight specific points in the amyloid peptide equilibrium that are highly sensitive to the ratio of Aβ40 to Aβ42, carry important implications for the pathogenesis and current therapeutic strategies of Alzheimer disease.

Alzheimer disease is a progressive neurodegenerative disorder characterized by age-related accumulation of amyloid-β (Aβ) proteins in the form of diffuse and neuritic plaques in regions of the brain that are affected by the disease (1–4). The discovery of Aβ fibrils as principal constituents of amyloid plaques led to the emergence of the amyloid hypothesis, which implicates the aggregation of Aβ as the primary trigger for a cascade of pathogenic events culminating in neurodegeneration and development of AD (1, 5–7). Aβ proteins are produced in neuronal and non-neuronal cells as a result of sequential proteolytic cleavage of the type I transmembrane amyloid precursor protein (APP) by β- and γ-secretases (8–12). Depending on the site of APP cleavage by γ-secretase, Aβ proteins of various chain lengths are generated (13–16). The predominant Aβ species in human plasma and CSF, as well as in conditioned media of APP-expressing cells, is Aβ40 (~90%) followed by Aβ42 (~10%). Despite the preponderance of Aβ40, in vivo studies reveal that Aβ42 is a major constituent of amyloid plaques and suggest that Aβ42 aggregation plays a critical role in the initiation of plaque formation and AD pathogenesis (17–20). In vitro, Aβ42 exhibits lower solubility and has the propensity to form protofibrils and fibrillar aggregates at lower concentrations and higher rates than Aβ40 or other Aβ variants (21–23). Aβ42 aggregates (protofibrils and fibrils) have also been reported to be more toxic to cultured neurons than Aβ40 aggregates (24, 25).

Although the majority of late-onset AD cases occur sporadically, genetic mutations in APP or subunits of γ-secretase (presenilins PS1 or PS2) account for a significant proportion of early-onset familial AD (FAD) cases (26, 27). Animal models and in vitro cell culture studies have shown that, in most instances, FAD mutations enhance total Aβ production, promote its aggregation and brain deposition, and/or alter the Aβ40/Aβ42 ratio in favor of Aβ42 production (28–30). Recent studies in human subjects also highlight the importance of Aβ40/Aβ42 ratio, rather than the total concentration of Aβ, as an important biomarker for AD progression and disease severity (31–33). To evaluate the consequences of altering the ratio Aβ40/Aβ42, several groups have investigated the effect of co-expressing the two Aβ variants (Aβ40 and Aβ42) or altering the expression level of one or the other variant in cellular and animal models of AD. These studies and other studies in human patients demon-

The abbreviations used are: Aβ, amyloid-β; AD, Alzheimer disease; FAD, familial Alzheimer disease; APP, amyloid precursor protein; PF, protofibrillar; M, monomeric; F, fibrillar; SF, sonicated fibrils; SEC, size exclusion chromatography; ThT, thioflavin T; TEM, transmission electron microscopy; PBS, phosphate-buffered saline.

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strate that the ratio of Aβ40 to Aβ42 is an important determinant of the distribution of amyloid pathology (i.e. parenchymal or vascular amyloid deposition) in the brains of patients with AD and transgenic AD mouse models (18, 34–37).

The molecular mechanisms by which changes in the Aβ40/Aβ42 ratio modulate the aggregation and toxicity of Aβ and influence the amyloid pathology distribution in the AD brain remain the subjects of considerable debate. Aβ40 inhibits fibril formation by Aβ42 (22, 38), and co-incubation of the two Aβ variants leads to formation of mixed prefibrillar aggregates in vitro (39). Aβ40 prevents Aβ42-induced neurotoxicity in cultured cells and in vivo (40), underscoring the regulatory effects of Aβ40/Aβ42 ratio on important events associated with Aβ aggregation and toxicity. More recently, Yan and Wang (41) used differential NMR isotope labeling to demonstrate that Aβ40 prevents aggregation of monomeric Aβ42 and is capable of being exchanged for Aβ42 monomer in Aβ42 aggregates.

In the present study, we determined the preferential effect of Aβ40 on the kinetic stability, solubility, and fibrillogenesis rate of specific aggregation states of Aβ42, including monomers, protofibrils, and fibrils. Additionally, we explored the dynamics of exchange between monomeric Aβ40 and Aβ42 at the end of protofibrils and fibrils formed by each peptide and determined the effect of these interactions on the aggregate growth and morphology in vitro. Finally, we examined the ability of Aβ42 fibrils and protofibrils to seed the aggregation of Aβ40 and vice versa by monitoring the seeding effects of homologous and heterogeneous sequence on the lag phase, elongation phase, and steady-state phase of fibril formation of monomeric Aβ40 and Aβ42. The specificity of interaction between Aβ40 and different Aβ42 aggregation states was validated by using Aβ40-1 (reverse) as a control peptide. The present work provides novel mechanistic and structural insights into the molecular mechanisms underlying the consequences associated with altered Aβ40/Aβ42 ratio. The implications of these findings for intervention strategies for AD are also discussed.

EXPERIMENTAL PROCEDURES

Chemicals and reagents of analytical grade were purchased from Sigma-Aldrich unless indicated otherwise. Best quality distilled water was used for preparation of buffers and solutions, which were filtered through 0.65-μm DVPP membranes (Millipore) before use.

Preparation of Protofibrillar and Monomeric Aβ

Aβ peptides 1-40, 1-42, and 40-1 were synthesized and purified by Dr. James I. Elliott at Yale University (New Haven, CT). Protofibrillar (PF) and monomeric (M) forms of Aβ were prepared according to the protocols described previously (42). Briefly, Aβ peptides were dissolved in 100% DMSO and adjusted to 1 mg/ml by adding distilled H2O. The pH of the resultant solutions was adjusted with 2 M Tris base, pH 7.4. After centrifugation (8000 × g at 4 °C for 10 min) the supernatant was injected into a size exclusion chromatography column Superdex 75 HR 10/30 (GE Healthcare) that had been equilibrated previously with 10 mM Tris-HCl, pH 7.4. Peptides were fractionated at a flow rate of 0.5 ml/min and eluted in 1.5-column volumes. Aβ elution was monitored by UV absorbance at three different wavelengths: 210, 254, and 280 nm. Under these conditions, Aβ42 eluted as two well separated peaks; one corresponding to the void volume of Superdex 75 containing Aβ protofibrils (Aβ42PF) and the second peak corresponding to monomeric Aβ (Aβ42M) (supplemental Fig. 1). Aβ40 and Aβ40-1 elute predominantly as single peaks corresponding to monomeric species (data not shown). The protein concentrations of the various Aβ fractions was estimated by UV absorbance at 280 nm in 10-mm path-length cuvettes using the theoretical molar extinction coefficient at 280 nm (1490 M⁻¹ cm⁻¹) (43) and/or using the Micro BCA protein assay (Pierce) when needed.

Fibrillogenesis Studies

Co-incubation of Monomeric Aβ40 with Protofibrillar or Monomeric Aβ42—Monomeric and protofibrillar preparations of Aβ42 and Aβ40 obtained by size exclusion chromatography (SEC) were adjusted to a final concentration of 10–20 μM in 10 mM Tris-HCl, pH 7.4, and placed in a 37 °C incubator without agitation. For co-incubation studies, protofibrillar and monomeric Aβ42 fractions were mixed with monomeric Aβ40 at molar ratios (Aβ42M:Aβ40M) of 10:1, 10:5, and 10:10 (all concentrations in μM) and allowed to aggregate at 37 °C without agitation. Fibril formation and protein solubility were monitored by thioflavin T (ThT) binding assay, negative staining transmission electron microscopy (TEM), and analytical SEC as described below. In parallel, the specificity of Aβ40 interactions with Aβ42 was validated by co-incubation with the control peptide Aβ40-1.

Fibril Elongation Studies—To probe the effect of monomeric Aβ on the elongation and reassociation of Aβ42 fibrils, we first generated Aβ42 fibrils by incubating 10 μM monomeric Aβ42 (Aβ42M) for 96 h. The fibrils were mechanically fragmented into smaller fibrillar structures (100–300 nm long) by ultrasonication on ice using a Vibra Cell™ instrument (Sonic Inc.) equipped with a 2-mm diameter microtip (20 × 5 s pulses; amplitude, 40%; output, 6 watts). The sonicated fibrils (Aβ42SF) were incubated: (i) in isolation; (ii) with monomeric Aβ40 (10 μM); (iii) with monomeric Aβ40 (10 μM) or (iv) with a 1:1 mixture of monomeric Aβ42:40 (20 μM final Aβ concentration). Fibril elongation and reformation of mature Aβ fibrils was monitored by ThT fluorescence and TEM.

Seeding Polymerization Studies

Seeding the Fibrillogenesis of Monomeric Aβ (Aβ40 and Aβ42) with Fibrils Derived from Aβ40 and Aβ42—To probe the ability of Aβ42 fibrils to accelerate the fibrillogenesis of monomeric Aβ40 and vice versa, 20 μM monomeric Aβ (Aβ42 or Aβ40) was incubated with the following: (i) 10 μg/ml sonicated Aβ42 fibrils (Aβ42SF, once); (ii) 20 μg/ml sonicated Aβ42 fibrils (Aβ42SF, twice); (iii) 10 μg/ml sonicated Aβ40 fibrils (Aβ40SF, once); and (iv) 20 μg/ml sonicated Aβ40 fibrils (Aβ40SF, twice). The extent of fibril formation was determined by ThT fluorescence and TEM. ThT fluorescence data were normalized by subtracting the contribution from sonicated fibrils.
Effect of Aβ40 on Fibrillation of Aβ42

Seeding the Fibrillogenesis of Monomeric Aβ (Aβ40 and Aβ42) with Aβ42 Protofibrils—20 μM monomeric Aβ (Aβ40 or Aβ42) was co-incubated with different molar ratios of protofibrillar Aβ42 (monomeric Aβ:Aβ42_Pf; 20:1, 20:2 and 20:4; all final concentrations in μM) and fibril formation was monitored by ThT fluorescence after subtracting the contribution from protofibrillar Aβ42. To probe the specificity of interactions between the monomeric and aggregated forms of Aβ42 and Aβ40, we also evaluated the capacity of sonicated fibrils and protofibrillar species of both peptides (Aβ40 and Aβ42) to seed the fibrillogenesis of Aβ40-1 (20 μM) in a similar fashion.

Thioflavin T Binding Assay

ThT binding assay was performed by mixing aliquots of 10–20 μM Aβ with 10–20 μM ThT dye (Aβ:ThT 1:1) and 50 mM glycine-NaOH, pH 8.5, in Nunc 384-well fluorescence plates (Fisher Scientific). ThT fluorescence of each sample was measured in an Analyst AF fluorometer (Molecular Devices) at excitation and emission wavelengths of 450 and 485 nm, respectively. The samples were analyzed in duplicates at selected time points.

Transmission Electron Microscopy

5 μl of sample was applied to carbon-coated Formvar 200 mesh grids (Electron Microscopy Sciences) and incubated at room temperature for 60 s. The grids were then washed sequentially by depositing 10-μl droplets of double distilled sterile water (2 times) followed by a 10-μl droplet of fresh 2% (w/v) uranyl acetate, which remained on the grid for 30 s. After each step, the excess solution was blotted with Whatman filter paper, and the grids were vacuum-dried from the edges. The samples were analyzed using a Philips CM-10 TEM microscope operated at 100 kV acceleration voltage.

Analytical Size Exclusion Chromatography

Analytical SEC was performed to quantify the relative amount of soluble (monomeric and protofibrillar) Aβ in solution at selected time points during the aggregation experiments. For this purpose a SEC column Superdex 75 PC 3.2/30 (GE Healthcare) was connected to a Waters Separation Module 2795 equipped with a photo diode array detector (Waters Corp.). Aliquots (150 μl) of the samples were centrifuged (8500 × g at 4 °C for 10 min), and 50 μl of supernatant was injected into the column. Samples were individually analyzed by UV absorption (wavelengths 210, 254, and 280 nm) at a flow rate of 0.05 ml/min.

Cell Culture Toxicity Studies

Primary Cell Cultures—Rat embryonic (E16) cortical cultures were established using a previously described procedure (44). Briefly, neurons were plated at a density of 30,000 cells/well in 96-well dishes (Costar™, Corning) previously coated with poly-L-lysine (Mr 30′000–70′000). On in vitro day 4, half of the medium was replaced with freshly prepared Neurobasal™ medium supplemented with 2% B27 (Invitrogen), 1× penicillin-streptomycin (Invitrogen), 0.5 mM l-glutamine, and 15 mM KCl. Subsequently, half of the medium was changed weekly. On in vitro day 23, half of the primary culture medium was replaced with complete Neurobasal medium containing one-fifth or one-tenth volume of amyloid peptide species with varying concentrations of Aβ40_M, Aβ42_M, Aβ42_PF, and Aβ42_F and 1:1 molar mixtures thereof. Cells were subsequently incubated with Aβ species for 7 days. All amyloid peptide species were delivered in 140 mM NaCl, 10 mM Tris, pH 7.4.

RESULTS

Isolation and Characterization of Protofibrillar and Monomeric Aβ—TEM images of protofibrillar Aβ42 (Aβ42_Pf) fractions revealed predominantly curvilinear structures with an average length and diameter of 60–100 and 4–6 nm, respectively (supplemental Fig. 1B). In addition, spherical aggregates of different diameters (6–10 nm) were also observed occasionally. The second elution peak corresponding to monomeric Aβ42 (Aβ42_M) did not show the presence of any recognizable aggregates by TEM (supplemental Fig. 1D). Unlike Aβ42, Aβ40 and Aβ40-1 (reverse) eluted predominantly as a single monomeric peak under the same solubilization conditions (data not shown) and did not form fibrils under the conditions used for Aβ42 fibrillogenesis studies (supplemental Fig. 2, A, C, and E). Fibril formation by monomeric Aβ40 (Aβ40_M) required gentle agitation (300 rpm) and higher concentrations (supplemental Fig. 2, B, D, and F).

Monomeric Aβ40 Inhibits the Fibrillogenesis of Monomeric Aβ42 in a Concentration-dependent Manner—To probe the effects of monomeric Aβ40 interactions on the self-assembly and fibril formation of monomeric Aβ42, we co-incubated SEC-isolated Aβ42_M with increasing concentrations of Aβ40_M (see “Experimental Procedures”). We found that Aβ40_M inhibited the fibrillogenesis of Aβ42_M in a concentration-dependent manner. After co-incubation for 96 h, strong (>50%) inhibition of monomeric Aβ42_M fibrillogenesis was observed in samples containing both peptides at Aβ40_M:Aβ42_M ratios ~ 0.5–1 (Fig. 1A). However, the presence of Aβ40_M, even at lower concentrations (Aβ40_M:Aβ42_M ~ 0.1) led to a transient population of...
protofibrillar species (Fig. 1B), which disappeared subsequently. In the absence of Aβ40, Aβ42M aggregated rapidly and formed long intertwining fibrillar networks without the accumulation of protofibrils (supplemental Fig. 1E). In contrast, co-incubation with Aβ40M favored the formation of short, flexible protofibrillar structures (Fig. 1, C and D, and Scheme 1), which did not appear to convert to mature elongated fibrils. Our data suggest that Aβ40M interferes with the ability of Aβ42M to form mature fibrils but does not interfere with its ability to form higher order prefibrillar aggregates. Under no conditions did we observe that mixtures of monomeric Aβ40 and Aβ42 remained as stable monomers or formed stable heterodimers on the time scale of our experiments (24–96 h).

Kinetic Stabilization of Aβ42 Protofibrils by Monomeric Aβ40—Next, we probed the interactions of monomeric Aβ (Aβ40 and Aβ42) with protofibrillar Aβ42 and assessed the consequences of such interactions on the elongation Aβ42 protofibrils into mature fibrils. For this purpose, we co-incubated Aβ42PF with monomeric Aβ (Aβ40M or Aβ42M) at different molar ratios (Aβ42PF:AβM, 10:1, 10:5, and 10:10; all final concentrations in μM) (Scheme 2). The addition of Aβ40M to Aβ42PF at increasing molar ratios resulted in enhanced ThT binding (Fig. 2A), consistent with the accelerated conversion of Aβ42PF into mature amyloid fibrils.

**FIGURE 1.** Monomeric Aβ40 inhibits fibril formation by monomeric Aβ42 in a ratio-dependent manner and affects the structure of amyloid aggregates. A, co-incubation of monomeric Aβ42 with increasing molar ratios of monomeric Aβ40 results in reduced ThT binding as an inverse function of monomeric Aβ40 concentration. The error bars represent mean ± S.D. in duplicate samples. M, monomeric Aβ; a.u., arbitrary units. B, SEC analysis on a Superdex 75 PC 3.2/30 column showing that soluble Aβ is transiently detected around 24 h of co-incubation and disappears subsequently, suggesting the formation of high molecular weight aggregates. Interestingly, co-incubation of monomeric Aβ42 with monomeric Aβ40 results in formation of various aggregate morphologies including short filamentous and protofibrillar structures. C and D, TEM images are shown for 10 μM monomeric Aβ42 incubated at 37 °C with 1 μM (C) and 10 μM monomer Aβ40 (D). Scale bar = 200 nm.

**SCHEME 1.** Monomeric Aβ40 inhibits the ability of Aβ42 to form mature amyloid fibrils. The arrows represent the relative concentration of each species.

**SCHEME 2.** Monomeric Aβ40 inhibits the conversion of Aβ42 into mature amyloid fibrils and favors the formation of curvilinear prefibrillar aggregates. The arrows represent the relative concentration of each species.
Equilibrium with monomers and/or a population of $A_{42\text{PF}}$ is more susceptible to dissociation upon interaction with the column matrix. After 24 h of co-incubation in the presence of $\geq 1 \mu M A_{40\text{M}}$, we observed a disappearance of the monomeric peak (Fig. 2D, 25 min), whereas the intensity and area of the protofibril peak increased (Fig. 2D, 18 min). Upon further incubation, the intensity and area of the protofibril peak was markedly reduced and exhibited a slight shift to higher molecular weight elution (16 min). These results suggest that the presence of $A_{40\text{M}}$ blocks the formation of mature $A_{42}$ fibrils but does not interfere with the growth of $A_{42}$ protofibrils and/or possibly leads to the formation of mixed $A_{40}/A_{42}$ protofibrillar aggregates. This hypothesis was confirmed by TEM studies that revealed protofibrillar clusters with a predominantly curvilinear morphology for all samples containing mixtures of $A_{42\text{PF}}$ and $A_{40\text{M}}$ regardless of the peptide ratios (Fig. 2F). The average length of these aggregates was variable and significantly larger than that of SEC-isolated $A_{42\text{PF}}$ (supplemental Fig. 1B). Of particular note, mature fibril structures similar to those formed by $A_{42}$ alone (Fig. 2E and supplemental Fig. 1E) were scarcely observed in samples containing mixtures of $A_{42\text{PF}}$ and $A_{40\text{M}}$.

Lack of Effect of $A_{40-1}$ on Monomeric or Protofibrillar $A_{42}$ Fibrillogenesis—To validate the specificity of the inhibitory effect of $A_{40}$ on the fibrillogenesis of monomeric and protofibrillar $A_{42}$, we co-incubated $A_{40\text{PF}}$ or $A_{40\text{M}}$ with $A_{40-1}$ at varying molar ratios ($A_{42}/A_{40\text{M}}$, 1:1, 10:5, and 10:10; all final concentrations in $\mu M$). The results of these studies show that $A_{40-1}$ does not inhibit fibril formation by protofibrillar $A_{42}$ at any of the concentrations tested (supplemental Fig. 3A). However, $A_{40-1}$ had a small inhibitory effect on $A_{42\text{M}}$ fibrillogenesis at a 1:1 ratio (supplemental Fig. 3B), but this activity was much less pronounced than the inhibitory affect of $A_{40\text{M}}$ (Fig. 1A). These results underscore the specificity of the interactions between the two endogenous $A\beta$ variants ($A_{40}$ and $A_{42}$) during amyloid fibril formation.

Monomeric $A_{40}$ Interferes with Growth and Reassembly of Preformed $A_{42}$ Fibrils— Whereas the above findings indicate that $A_{40}$ inhibits de novo fibrillogenesis of monomeric and protofibrillar $A_{42}$, we next sought to explore the possible
Effect of Aβ40 on Fibrillization of Aβ42

The presence of Aβ in isolation (A) generated fibrils. Aβ42 fibrils were mechanically disrupted by ultra-sonication (B) and resulted in fragmented fibrils (C) that were mechanically disrupted by sonication (Aβ42SF). The addition of monomeric Aβ40 (at a 1:1 molar ratio to sonicated Aβ42SF) resulted in fibril elongation (D). Scale bar = 200 nm. The error bars in A represent the mean ± S.D. in duplicate samples. M, monomeric Aβ; F, fibrillar Aβ42; SF, sonicated Aβ42 fibrils; a.u., arbitrary units.

For this purpose, Aβ42 protofibrils (10 μM) were incubated in the presence of Aβ42M (10 μM), Aβ40M (10 μM), or a 1:1 mixture of both peptides (20 μM final Aβ concentration) for 72 h (Fig. 9). After this, a second 10 μM addition of freshly prepared monomeric Aβ species (Aβ42M, Aβ40M, or Aβ42M plus Aβ40M) was carried out, and the resultant mixtures were incubated for an additional 78 h (150 h total) (Scheme 4). At each step of the process, the presence of fibrils was monitored by ThT binding and negative staining TEM. As expected, the addition of Aβ42M to Aβ42PF resulted in enhanced ThT fluorescence (Fig. 4A, II, 72 h) and the formation of mature fibrillar structures (Fig. 3, A and D), whereas the addition of Aβ40M retarded their growth and blocked fibril reassembly as evidenced by significantly reduced ThT fluorescence (Fig. 3A). TEM images of the fragmented fibrils incubated in the presence of Aβ40M also revealed predominantly truncated fibrillar aggregates and the absence of mature fibrils (Fig. 3E). Co-incubation of sonicated Aβ42 fibrils with a 1:1 mixture of Aβ42M:Aβ40M resulted in fibril growth and reassembly but to a lesser extent than seen after Aβ40M addition (data not shown). These observations suggest that Aβ40, at near equimolar ratios, retards the elongation and maturation of fragmented Aβ42 fibrils into classical ThT-binding amyloid fibrils (as shown in Figs. 2E and 3B and supplemental Fig. 1E).

Monomeric Aβ42 and Aβ40 Are Constantly Recycled and Compete for Binding to the Ends of Protofibrillar and Fibrillar Aβ Aggregates—The above observations highlight a significant degree of molecular cross-talk between Aβ40 and different aggregation states of Aβ42 (monomer, protofibrils, and fibrils) during amyloid fibril formation. Hence, we ventured to further explore this phenomenon and probe the dynamics of exchange between monomeric Aβ42 and Aβ40 at the end of protofibrillar and fibrillar forms of Aβ42. For this purpose, Aβ42 protofibrils (10 μM) were incubated in the presence of Aβ42M (10 μM), Aβ40M (10 μM), or a 1:1 mixture of both peptides (20 μM final Aβ concentration) for 72 h (Fig. 9). After this, a second 10 μM addition of freshly prepared monomeric Aβ species (Aβ42M, Aβ40M, or Aβ42M plus Aβ40M) was carried out, and the resultant mixtures were incubated for an additional 78 h (150 h total) (Scheme 4). At each step of the process, the presence of fibrils was monitored by ThT binding and negative staining TEM. As expected, the addition of Aβ42M to Aβ42PF resulted in enhanced ThT fluorescence (Fig. 4A, II, 72 h) and the formation of mature fibrillar structures (Fig. 3, A and D), whereas the addition of Aβ40M retarded their growth and blocked fibril reassembly as evidenced by significantly reduced ThT fluorescence (Fig. 3A). TEM images of the fragmented fibrils incubated in the presence of Aβ40M also revealed predominantly truncated fibrillar aggregates and the absence of mature fibrils (Fig. 3E). Co-incubation of sonicated Aβ42 fibrils with a 1:1 mixture of Aβ42M:Aβ40M resulted in fibril growth and reassembly but to a lesser extent than seen after Aβ40M addition (data not shown). These observations suggest that Aβ40, at near equimolar ratios, retards the elongation and maturation of fragmented Aβ42 fibrils into classical ThT-binding amyloid fibrils (as shown in Figs. 2E and 3B and supplemental Fig. 1E).
Effect of Aβ40 on Fibrillization of Aβ42

![Graph showing ThT fluorescence over time](image)

**FIGURE 4.** Monomeric Aβ40 and Aβ42 exchange along different stages of amyloid formation imparts distinct structural features on amyloid aggregates. Aβ42 protofibrils were exposed to sequential addition of either monomeric Aβ42 or monomeric Aβ40 for an extended period of time (A) (see “Experimental Procedures” and Fig. 8 for details). When added to Aβ42 fibrils, monomeric Aβ40 alters the fibrillar structure (B) and leads to the emergence of curvilinear morphologies (C). Moreover, the addition of monomeric Aβ42 to Aβ40-stabilized protofibrils (D) results in conversion of protofibrillar structures into elongated fibrils (E). When added to Aβ42 protofibrils, a 1:1 mixture of monomeric Aβ42 and Aβ40, delays the emergence of mature fibrils (72 h (F) and 150 h (G)). Scale bar = 200 nm. The error bars in A represent the mean ± S.D. in duplicate samples. M, monomeric Aβ; PF, protofibrillar Aβ42; a.u., arbitrary units.

III, 72 h), and non-fibrillar structures resembling protofibrils were observed as the predominant species (Fig. 4D). However, the addition of Aβ42M overcame the stabilizing effect of Aβ40 on protofibrils, resulted in enhanced ThT fluorescence (Fig. 4A, III, 150 h), and led to emergence of long mature fibrils (Fig. 4E). Finally, serial additions of a 1:1 mixture of monomeric Aβ42SI: Aβ40M to Aβ42 protofibrils resulted in a slight rise in ThT fluorescence relative to that obtained upon the addition of Aβ40M alone (Fig. 4A, IV). An examination of this sample by TEM revealed mainly curvilinear non-fibrillar structures and short protofibrils (Fig. 4F, G and H). The observation that Aβ40M can alter the structure of preformed Aβ42, supports the notion that Aβ40 and Aβ42 monomers are exchangeable at the growing ends of amyloid aggregates (protofibrils and fibrils).

**SCHEME 5.** Aβ42 fibrils nucleate the fibrillogenesis of monomeric Aβ40 and vice versa.

**Aβ42 Fibrils Nucleate the Fibrillogenesis of Monomeric Aβ40 and Vice Versa**—Previous studies have established the ability of Aβ fibrils to induce fibril formation by monomeric Aβ through the process of nucleated polymerization (22, 38). Thus, we sought to explore the ability of both well characterized protofibrillar and fibrillar forms of Aβ42 to seed the fibrillogenesis of monomeric Aβ40 and Aβ42. For these experiments, we isolated aggregate-free monomeric Aβ40 and Aβ42 by SEC and characterized its fibrillogenesis in the presence of different amounts of fibrillar seeds of either Aβ40 or Aβ42 (sonicated fibrils of Aβ42sF at 10 and 20 μg/ml and sonicated fibrils of Aβ40sF at 10 and 20 μg/ml) (Scheme 5). Aβ42sF (20 μM) alone (with no seeds) exhibited an appreciable rise in ThT binding after 15 h and reached a plateau after 50 h of incubation at 37 °C. The addition of sonicated fibrils of Aβ42sF abolished the lag
phase and reduced the time required to reach near equilibrium levels of fibril formation (~9–15 h) (Fig. 5A). Interestingly, addition of sonicated fibrils of Aβ42SF also decreased lag phase time but exhibited a longer time to reach near equilibrium (~25–30 h) (Fig. 5A). Regardless of the seeding species, Aβ42M forms similar fibrils that are ≥1 μm long and have diameters of 10–14 nm. These fibrils appear to be composed of 2–3 protofilaments and exhibit a helical twist spaced at 60–180 nm (Fig. 5, E and F). For the purpose of discussion, we will refer to these fibrils as type I Aβ fibrils.

In parallel, we investigated the ability of Aβ40SF and Aβ42SF to seed the fibrillogenesis of monomeric Aβ40. For this purpose, Aβ40M (20 μM) was co-incubated with Aβ42SF (10 and 20 μg/ml) and Aβ40SF (10 and 20 μg/ml). It is noteworthy that...
Effect of Aβ40 on Fibrillization of Aβ42

Aβ40M (20 μM) alone (with no seeds), under stagnant conditions, did not form amyloid fibrils during the time scale of these experiments (Fig. 5B and supplemental Fig. 2, A and E) but formed fibrils readily at higher concentrations or under agitating conditions (supplemental Fig. 2, B and F). When Aβ40SF or Aβ42SF was added to the solution of monomeric Aβ40, we observed two distinct effects on Aβ40 assembly: (i) the elongation phase was sensitive to the concentration of seeding fibrils, and (ii) at all time points, the level of ThT binding near equilibrium was relatively higher in the presence of Aβ40SF than of Aβ42SF, suggesting preferential seeding in the presence of Aβ40SF (Fig. 5B). Moreover, Aβ40M generated similar fibrils regardless of the nature and amount of seeding species (Fig. 5, G and H). The resulting fibrils had variable lengths with diameters of 6 to 14 nm. The fibrils formed by Aβ40 consisted of ≥3 protofilaments and exhibited a helical periodicity of 110 to 180 nm. TEM analysis of a 20 μM Aβ40M sample incubated under identical conditions for 100 h did not show any fibrils (data not shown).

Aβ42 Protofibrils Seed the Fibrillogenesis of Monomeric Aβ42 but Not Monomeric Aβ40—To explore the ability of Aβ42 protofibrils to seed the fibrillogenesis of monomeric Aβ (Aβ40 and Aβ42), we co-incubated 20 μM monomeric Aβ (Aβ40M or Aβ42M) with three different molar ratios of Aβ42PF (i.e. Aβ3M: Aβ42PF at 20:1, 20:2, and 20:4; all final concentrations in μM). We found that even the lowest concentration of Aβ42PF (1 μM) was sufficient to accelerate the fibrillogenesis of monomeric Aβ42 (Fig. 6A), whereas no such effect was observed (for any molar ratio) when Aβ42PF were added to monomeric Aβ40 under identical conditions (Fig. 6B). TEM analysis of these co-incubation samples confirmed the validity of the above mentioned observations such that when protofibrillar Aβ42 was added to monomeric Aβ42, extended networks of fibrillar structures were observed at all molar ratios (Aβ42M:Aβ42PF, 20:4 μM (Fig. 6C)). In contrast, the addition of Aβ42PF to Aβ40M resulted in the formation of mainly short flexible non-fibrillar structures (Aβ40M:Aβ42PF, 20:4 μM (Fig. 6D)). The absence of mature Aβ40 fibrils indicates that Aβ42 protofibrils are less efficient as seeding nuclei for Aβ40 fibrillogenesis as compared with fibrillar Aβ42.

In parallel, we co-incubated 20 μM Aβ40-1 with sonicated fibrils of Aβ (Aβ40SF or Aβ42SF, 20 μg/ml) or protofibrillar Aβ42PF (4 μM) as control experiments. Neither fibrillar Aβ (Aβ40SF and Aβ42SF) nor protofibrillar Aβ42PF resulted in fibril formation from Aβ40-1 (supplemental Fig. 5).

Treatment of Cultured Neurons with Specific Aggregation-prone Aβ Species or Mixtures Is Associated with Decreased Neuronal Viability—To assess the differential effects of monomeric Aβ (Aβ40 or Aβ42) on the toxicity of Aβ42 aggregates to pri-
Effect of Aβ40 on Fibrillization of Aβ42

In the present study, we explored the interactions of distinct aggregation states of each peptide influence Aβ aggregation, fibril formation, and cellular toxicity. In the present work, we have uncovered new aspects of Aβ aggregation equilibria by investigating the ratio-dependent effects of monomeric Aβ40 on the fibrillogenesis of monomeric and protofibrillar forms of Aβ42 and the dynamic reassembly of short Aβ42 fibrils. By studying well defined aggregation states of Aβ, our findings provide critical new mechanistic insights about how Aβ40 and Aβ42 interact at different stages along the amyloid formation pathway. These findings have allowed us to develop a new working model of Aβ42 and Aβ40 interactions, which could serve as mechanistic explanation for in vivo amyloid formation (Fig. 9).

The Ratio of Aβ42 Aggregates to Monomeric Aβ40 Is an Important Determinant of Aβ Aggregation and Fibrillogenesis—

The in vitro studies presented here demonstrate that maintain-
Effect of Aβ40 on Fibrillization of Aβ42

FIGURE 8. The ratio of monomeric (Aβ40 and Aβ42) to aggregated forms of Aβ40 and Aβ42 is an important determinant of Aβ aggregation and fibrillogenesis. This is a schematic illustration of the effect of monomeric Aβ40 on the fibrillogenesis of the monomeric (A), protofibrillar (B), and fibrillar (C) forms of Aβ42. Aβ40 and Aβ42 species (M, PF, and F) are depicted in grey and black, respectively. The level of ThT dye binding and fluorescence observed for the final aggregates formed in each of the experiments listed is indicated by plus signs: ++++++ is indicative of high ThT fluorescence, and + is indicative of low ThT fluorescence.

ing a delicate balance not only between the monomeric forms of Aβ42 and Aβ40 but also between the monomeric and aggregated forms of both peptides is essential for maintaining Aβ solubility and preventing Aβ fibrillogenesis and toxicity. First, in the presence of excess Aβ40M (as is expected under normal physiological conditions), Aβ42M aggregation is discouraged because of its low concentration and the inhibitory effect of excess Aβ40M. As the concentration of Aβ42M increases (due to FAD-associated mutations in APP or other local factors), the formation of high molecular weight protofibrillar aggregates or low ThT-binding fibrillar structures is favored (Fig. 8). The molecular size, kinetic stability, and potential toxicity of such species depend on the local concentration of Aβ40 at the site of their formation or localization. The stabilization of protofibrillar and/or low ThT-binding Aβ aggregates could explain the lack of clear correlation between amyloid plaque pathology and cognitive decline in patients with AD (53–55). Figs. 8 and 9 summarize the effect of the ratio of monomeric (Aβ40 and Aβ42) to aggregated forms of Aβ40 and Aβ42 in the structural and ThT binding properties of the resultant Aβ aggregates. At higher Aβ42M/Aβ40M ratios, amyloid fibril formation is favored and can be accelerated by seeding with fibrils of either Aβ40 or Aβ42. At Aβ40M/Aβ42 ratios of 0.5 to 1, the formation of mature amyloid fibrils is inhibited, and predominantly protofibrillar and/or low ThT binding fibrils are formed. Interestingly, Aβ42 fibrils seed the fibrillogenesis of both peptides (Aβ40 and Aβ42); however, Aβ42 protofibrils were found to seed the fibrillogenesis of monomeric Aβ42 but not of monomeric Aβ40. These observations support the notion that Aβ42 is more highly fibrillogenic and plays a crucial role in the initiation and progression of Aβ aggregation.

Aβ42 Fibril Growth and Reassembly Is Modulated by Constant Recycling and Competition between Monomeric Aβ40 and Aβ42—Previous studies have shown that amyloid fibril growth occurs by monomer addition to the growing fibril ends (56–59). In addition, NMR and mass spectrometry studies have also shown that monomeric subunits constantly recycle in and out of the fibrillar backbone (60, 61). Given our findings that Aβ40 inhibits the fibrillogenesis of monomeric and protofibrillar Aβ42, we hypothesized that Aβ40 also intervenes in the growth and reassembly of Aβ42 fibrils. We envisioned that this could occur by several mechanisms, two of them being: (i) by competing at the free ends of Aβ42 fibrils and preventing addition of monomeric Aβ42; or (ii) by interacting and sequestering free monomeric Aβ42 in solution and preventing its docking back into Aβ42 fibrils. To test these two possibilities, we examined the effect of Aβ40 on the growth and reassembly of fragmented Aβ42 fibrils (see “Experimental Procedures”). In the presence of excess monomeric Aβ42, the fragmented Aβ42 fibrils grew and reassembled rapidly to form fibrillar structures identical to that of the parent fibrils (Figs. 3B and 9). In contrast, the addition of monomeric Aβ40 retarded Aβ42 fibril reassembly, leading to the accumulation of truncated fibrillar structures that exhibited low ThT binding and did not convert to mature fibrils in our experimental time scale (up to
Effect of Aβ40 on Fibrillization of Aβ42

Distribution in Vivo—The transient formation of protofibrils during the fibrillogenesis of almost all amyloid forming proteins supports the notion that protofibrils are obligate intermediates that are likely to exist, at least transiently, in vivo. Aβ protofibrils and oligomers (dimers, trimers, up to dodecamers) have been prepared in vitro from synthetic peptides or purified from conditioned media of APP-overexpressing cells (62, 63), are biologically active entities (64), and bind, albeit weakly, to the amyloid-specific dyes such as ThT and Congo red (65). Several independent studies have implicated Aβ protofibrils as the predominant toxic species through effects that include inhibition of long term potentiation (64, 66), disruption of signal transduction (67), and formation of channels or pores in cellular membranes (68–70). These findings have led to the hypothesis that conversion of small toxic Aβ aggregates into fibrils, and their sequestration within inclusions or plaques, is neuroprotective and may represent an active natural detoxification mechanism (71). Hence, it can be argued that factors that alter the distribution and/or kinetic stability of Aβ protofibrils should influence their aggregation and toxicity in vivo. Therefore, stabilizing a toxic population of protofibrils should enhance toxicity and accelerate disease progression. In contrast, stabilization of a nontoxic protofibrillar or fibrillar forms of Aβ may have beneficial effects. In fact, a recent report suggests that reducing the lifetime of protofibrils by accelerating their conversion into amyloid fibrils protects against Aβ induced toxicity (72).

Our study demonstrates that Aβ40 enhances the kinetic stability of Aβ42 protofibrils and, at high concentrations, promotes the formation of low ThT-binding aggregates. This could provide possible mechanistic explanations for the effect of the ratio of Aβ40 to Aβ42 on the distribution of amyloid pathology (parenchymal versus vascular). In the presence of high Aβ40/Aβ42 ratios (> 10), Aβ fibril formation is accelerated and leads to local accumulation of fibrillar aggregates in brain parenchyma. In contrast, subphysiologic Aβ40/Aβ42 (monomeric or protofibrillar) ratios (0.5–2) favor the accumulation and persistence of soluble Aβ aggregates (protofibrils and other non-fibrillar structures). Being relatively soluble in nature, such aggregates can be transported across different brain compartments, including the vasculature, where they can fibrillize if encountered by supraphysiologic concentrations of monomeric Aβ42 and Aβ40. This model supports a critical role for Aβ42 role in initiating and accelerating Aβ40 fibrillization in the vasculature, as has also been reported (19, 73).

Fibrillogenesis of Aβ Contributes to Aβ Neurotoxicity—To establish the neurotoxic activities of individual and mixed Aβ...
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(Aβ40 and Aβ42) monomers and aggregates, we treated cultured neurons with defined aggregate states of Aβ40 (protofibrils and fibrils) with or without the addition of monomeric Aβ (Aβ40 or Aβ42). Comparative analysis of NeuN positivity revealed that neither monomeric Aβ (Aβ40 or Aβ42) nor aggregated Aβ42 (protofibrillar or fibrillar) was sufficient to impair neuronal viability at lower (0.1 and 5 μM) concentrations (Fig. 7A and supplemental Fig. 6). Furthermore, adding monomeric Aβ40 to Aβ42 (monomeric, protofibrillar, or fibrillar; 5 μM each) did not enhance Aβ toxicity (Fig. 7, A and D). However, the addition of monomeric Aβ40 to protofibrillar Aβ42 (5 μM each) or 10 μM monomeric Aβ42 significantly impaired neuronal viability (Fig. 7, A, C, and E), suggesting that perpetuating the aggregation of Aβ42 increases Aβ toxicity. Interestingly, a higher concentration (10 μM) of protofibrillar Aβ42 or preformed Aβ42 fibrils did not show comparable toxicity (Fig. 7, A and G). These data are consistent with a dominant role for Aβ42 in impairing neuron viability (19, 25) and further implicate the ongoing Aβ self-assembly process (rather than any specific Aβ aggregation state) in Aβ-associated toxicity (51, 74). These results also add credence to therapeutic strategies aimed at disrupting the nucleation or elongation of amyloid fibrils to inhibit Aβ neurotoxicity in AD.

Conclusions—Potential interactions between Aβ40 and Aβ42 have been investigated previously by various research groups. These studies have shown that Aβ40 can inhibit the aggregation and fibril formation of Aβ42 (38, 41) and protect cultured neurons from Aβ-induced neurotoxicity (40). In addition to confirming the findings of these studies, the present study serves as a critical contribution to the current understanding of Aβ40 and Aβ42 interactions by examining the specific interplay of biophysically defined Aβ species (and mixtures thereof). Unlike previous studies suggesting that Aβ40 blocks the aggregation of monomeric Aβ42 (41), our results show that Aβ40 does not prevent further aggregation of the various Aβ42 quaternary structures (monomeric, protofibrillar, and fibrillar). Instead, we have demonstrated that Aβ40 alters the kinetic stability, solubility, and morphological properties of Aβ42 aggregates and prevents the formation of mature fibrils. Accordingly, we found that Aβ40 inhibits fibril formation of monomeric Aβ42 in a concentration-dependent manner (Fig. 1), whereas the effect on protofibril and fibril elongation is concentration-independent (Fig. 2). We have also shown that changes induced by Aβ40 are reversible upon reintroduction of high concentrations of Aβ42. Our results also confirm previous findings on the seeding capacity of Aβ40 and Aβ42 fibrils (Fig. 5), but to our knowledge this is the first report showing that Aβ42 protofibrils have the capacity to enhance the fibrillogenesis of Aβ42 but not of Aβ40 (Fig. 6D). Importantly, we have also begun to establish the relevance of the interactions of specific Aβ species in vivo. These results suggest that the continued pursuit of a quantitative and structural understanding of how Aβ40 and Aβ42 interact may eventually provide better biomarkers and therapies for the treatment of AD.

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