Temporal Profile of Amyloid-β (Aβ) Oligomerization in an in Vivo Model of Alzheimer Disease

A LINK BETWEEN Aβ AND TAU PATHOLOGY*†‡§¶

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Accumulation of amyloid-β (Aβ) is one of the earliest molecular events in Alzheimer disease (AD), whereas tau pathology is thought to be a later downstream event. It is now well established that Aβ exists as monomers, oligomers, and fibrils. To study the temporal profile of Aβ oligomer formation in vivo and to determine their interaction with tau pathology, we used the 3xTg-AD mice, which develop a progressive accumulation of plaques and tangles and cognitive impairments. We show that SDS-resistant Aβ oligomers accumulate in an age-dependent fashion, and we present evidence to show that oligomerization of Aβ appears to first occur intraneuronally. Finally, we show that a single intrahippocampal injection of a specific oligomeric antibody is sufficient to clear Aβ pathology, and more importantly, tau pathology. Therefore, Aβ oligomers may play a role in the induction of tau pathology, making the interference of Aβ oligomerization a valid therapeutic target.

Alzheimer disease (AD)† is the most common neurodegenerative disorder, affecting ~5 million Americans (1). Neuropathologically, it is characterized by the accumulation of extracellular plaques, mainly comprised of a small peptide called amyloid-β (Aβ), and intracellular neurofibrillary tangles, consisting of aggregates of hyperphosphorylated tau protein (2). Based on compelling genetic evidence, it has been postulated that pathological assemblies of Aβ are the cause of all forms of AD (3), whereas tau pathology and other neuropathological changes are a downstream consequence of the pathological accumulation of Aβ species. This hypothesis has received strong experimental support from studies of various transgenic models of AD (4–6). The source of Aβ that initiates the neurodegenerative process, however, remains unknown. Traditionally, Aβ has been viewed as being generated and secreted extracellularly, but it is also becoming increasingly apparent that some Aβ can be generated in different intracellular compartments, such as the endoplasmic reticulum and the trans-Golgi (7–11). Moreover, there is mounting evidence to support a pathophysiologic role for intracellular Aβ in AD an Down syndrome (see Ref. 12 for review).

Aβ exists in several different physical states, including as monomers, oligomers, or fibrils. Evidence from in vitro studies demonstrates that synthetic Aβ monomers aggregate in a time-dependent fashion to form oligomers, which eventually may form fibrils (13–15). During the last decade, in vitro and in vivo experimental evidence points to soluble Aβ oligomers, also referred to as Aβ-derived diffusible ligands, as the predominant neurotoxic species for neurons (16, 17). In this regard, Aβ oligomers are very potent toxic species, as even nanomolar concentrations have been shown to kill mature neurons in hippocampal slices (18). Moreover, Aβ oligomers appear to interfere with many critical neuronal activities, including inhibiting long term potentiation (LTP) in organotypic hippocampal slices (18, 19). Aβ oligomers can also cause calcium dysregulation and membrane disruption, thus interfering with overall cell functioning (20, 21). The toxicity of Aβ oligomers has also been shown in vivo. In particular, intracerebroventricular injection of oligomers inhibits LTP (22) and specifically disrupts cognitive function (23). Importantly, the concomitant injection of the anti-Aβ antibody 6E10 with Aβ oligomers neutralizes the oligomer-induced LTP dysfunction (24). These data strongly support the idea that oligomers represent a fundamental species responsible for mediating Aβ toxicity in AD (2, 16).

Given the critical role that Aβ oligomers appear to play in the pathogenesis of AD, we sought to understand their genesis in an in vivo model of AD. We used the 3xTg-AD mice, which develop an age-dependent accumulation of both plaques and tangles in AD-relevant brain regions (25). We first charted the temporal relationship between Aβ monomers, oligomers, and fibrils formation. Of particular relevance, we found that oligomerization of Aβ appears to commence intraneuronally in the 3xTg-AD brains, a finding consistent with other in vitro and in vivo studies (26–28). As we previously showed that the administration of Aβ-specific antibodies reduces not only the Aβ pathology but also early forms of tau pathology (6), here we extended these studies and showed that the administration of an Aβ oligomeric-specific antibody is also able to reduce the tau pathology in these mice. These findings suggest that the action of Aβ oligomers may be a key event in the initiation of the tau pathology.

MATERIALS AND METHODS

Mice—We previously described the generation of the 3xTg-AD mice (25). Briefly, the 3xTg-AD mice were derived by co-microinjecting two independent transgenes encoding human APPSwe and the human tauP301L (both under control of the mouse Thy1.2-regulatory element) into single-cell embryos harvested from homozygous mutant PS1M146V knock-in (PS1-KI) mice.

Immunoblotting—Brains from 3xTg-AD and NonTg mice were homogenized in 2% SDS supplemented with a complete miniprotease inhibitor tablet (Roche Diagnostics). The homogenized mixtures were

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2 The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid-β; LTP, long term potentiation.
briefly sonicated to sheer the DNA and centrifuged at 4 °C for 1 h at 100,000 × g. The supernatant was used for dot blot and Western blot. Protein concentration was determined using the Bio-Rad protein assay, and samples were adjusted with 2% SDS to the same concentration. For the dot blot, brain extracts were applied to a nitrocellulose membrane and air-dried. For the Western blot, brain extracts were heated in loading buffer (NuPAGE LDS sample buffer from Invitrogen) for 10 min at 70 °C and resolved by SDS-PAGE (10% Bis-Tris from Invitrogen) under reducing conditions and transferred to a nitrocellulose membrane. Membranes were incubated in a 5% solution of nonfat dry milk for 1 h at 20 °C. After overnight incubation at 4 °C with primary antibody, the blots were washed in Tween 20-TBS (T-TBS) (0.02% Tween 20, 100 mM Tris, pH 7.5, 150 mM NaCl) for 20 min and incubated at 20 °C with the appropriate secondary antibody for 1 h. The blots were washed in T-TBS for 20 min and incubated for 5 min with Super Signal (Pierce), washed, and exposed.

**Immunohistochemistry**—Brains from 3xTg-AD and NonTg mice were fixed for 48 h in 4% paraformaldehyde. Free-floating sections (50-μm thick) were obtained using a vibratome slicing system (Pelco, Redding, CA) and stored in 0.02% sodium azide in phosphate-buffered saline. The endogenous peroxidase activity was quenched for 30 min in 3% H2O2. Sections were then incubated in 90% formic acid for 7 min to expose the epitope. The appropriate primary antibody was applied overnight at 4 °C. Sections were washed with TBS and incubated with the appropriate secondary antibody for 1 h at 20 °C. Sections were developed with diaminobenzidine substrate using the avidin-biotin horseradish peroxidase system (Vector Laboratories, Burlingame, CA). For confocal microscopy, after incubating the sections in the appropriate primary antibody, suitable Alexa Fluor secondary antibody (Molecular Probes) was applied. Subsequently, sections were washed in phosphate-buffered saline, mounted on slide and coverslip with Vectashield (Vector Laboratories).

**RESULTS**

The development of oligomeric-specific antibodies has greatly facilitated the understanding of the role of oligomers in the pathogenesis AD. In this study we used antibody M71/3, which is highly sensitive and detects minute amounts of low molecular weight AB oligomers (12–24mers) (29, 30), and antibody A11, which is selective for higher molecular weight AB oligomers (31). Neither of these antibodies cross-react with AB monomers or natively folded amyloid precursor protein (29, 31).

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the resultant proteins were applied to a nitrocellulose membrane, which was subsequently probed with the oligomer-specific antibodies M71/3 and A11. At 2 months of age, the signal intensity was comparable between the 3xTg-AD and NonTg samples, suggesting that Aβ oligomers have not yet built up to any significant extent (Fig. 1A). Although the NonTg signal remained low across all time points, there was an age-dependent increase in M71/3- and A11-specific immunoreactivity in the 3xTg-AD samples, first apparent by 6 months of age (Fig. 1, A and B). Thus, oligomerization of Aβ starts between 2 and 6 months of age. Interestingly, we found that the intensity of both M71/3 and A11 immunoreactivity was significantly lower at 9 and 12 months of age compared with the 6 months time point (Fig. 1, A and B). Although the reason for this decrease in oligomer levels during these ages is not clear, it may be because of an increase in Aβ fibrilization that occurs at these ages (see below). This transient reduction occurs within a narrow time frame as between 12 and 20 months, we again found an age-dependent increase in Aβ oligomers in the 3xTg-AD brains (Fig. 1, A and B).

Aβ oligomers appear to be relatively stable structures, as SDS-resistant species have been isolated from post-mortem human brains and cell lines (22, 29, 32, 33). We determined whether SDS-resistant Aβ oligomers could also be isolated from the 3xTg-AD brains and resolved by SDS-PAGE and Western blotting. Following preparation of protein extracts in 2% SDS, we detected SDS-resistant Aβ dimers, trimers, and pentamers in brain samples from 20-month-old mice by Western blot using 6E10 (Fig. 1C). We were unable to detect Aβ (monomeric or oligomeric) in younger mice using this particular immunoblotting approach, perhaps because Aβ oligomers are not yet SDS-resistant and/or their presence is below threshold for detection by a Western blot. These data indicate that the SDS-resistant oligomers build-up in an age-dependent fashion in the 3xTg-AD mice and are readily detectable in older mice.

Results from several in vitro studies suggest that Aβ oligomers may be a transient intermediate stage leading to plaque formation; however, recent studies have also raised the possibility that Aβ oligomers could be more stable and not convert into fibrils (34). To determine the temporal relationship between these two Aβ species in vivo, we charted their accumulation in the hippocampus of the 3xTg-AD mice using antibody 6E10 (which detects all forms of Aβ), M71/3 (which detects low molecular weight Aβ oligomers at the fentomole levels) (30), and thioflavine S staining (to identify fibrillar Aβ deposits). We focused on the hippocampus because this brain region is highly affected by Aβ pathology in both AD and in the 3xTg-AD mice; moreover, the well defined structural organization of the hippocampus facilitates the study of the temporal progression of Aβ aggregation. At 4 months of age, robust intraneuronal staining was apparent with antibody 6E10, but not with M71/3 or thioflavine S (Fig. 2, A–C), suggesting that oligomerization of Aβ occurs intraneuronally between 4–6 months of age (see also Fig. 3A). Notably, by 12 months, intraneuronal Aβ oligomers are no longer apparent in the CA1/subiculum, although we found a marked increase in M71/3 extracellular staining (c.f. Fig. 2, I and H). Because the M71/3 antibody does not discriminate between oligomers and fibrils, we cannot exclude the possibility that some of the positive material...
shown in Fig. 2H is fibrillar in nature. However, staining of an adjacent section with thioflavine S (Fig. 2J) reveals far less staining compared with that detected by M71/3, suggesting that the majority of the extracellular deposits shown in Fig. 2H is most likely extracellular Aβ oligomers. Thus, there is an age-dependent shift in M71/3 immunoreactivity in the CA1/subiculum region from predominantly intracellular to predominantly extracellular staining between 6 and 12 months. Please note that protein samples shown in the dot blot in Fig. 1 were prepared in 2% SDS, which may have disrupted some Aβ structures, thereby accounting for the lower levels at this time point compared with the immunohistochemistry data. The presence of extracellular Aβ oligomers appears to occur prior to extracellular thioflavine-positive plaques (Fig. 2), providing correlative in vivo evidence that oligomers represent an intermediate step leading to plaque formation. However, these data do not exclude the possibility that a fraction of Aβ oligomers can be more stable structures or that these structures can be independent of fibrillarization.

To better analyze the conformation of intraneuronal Aβ oligomers, we double labeled sections from the 3xTg-AD mice with M71/3 and Aβ42-specific antibodies and analyzed them using confocal microscopy. We found that the oligomeric Aβ staining in the soma was very punctuate, indicating that these structures are located in an intracellular compartment (Fig. 3A). A similar staining pattern was detected with the Aβ42-specific antibody (Fig. 3B). We found that only a proportion of deposits detected by the Aβ42-specific antibody were also immunoreactive for M71/3, as Aβ-specific antibodies recognize monomeric Aβ as well (Fig. 3C). Surprisingly, however, we found a proportion of M71/3-positive structures that were negative for Aβ-specific antibodies (Fig. 3C). These data suggest that intraneuronal oligomers exist in different conformations, one where the C-terminal of Aβ is accessible, and one where it is not. Original magnification for A–C is 80× and for D–F is 20×.

Co-localization of Aβ Oligomers with Early, Somatodendritic Tau but Not Late Hyperphosphorylated Tau—Recent evidence suggests that Aβ and tau pathology may influence each other. As part of our efforts to discern the nature of this interaction and to establish which form of Aβ may be involved, we first determined whether there was co-localization between Aβ oligomers and tau in vivo. Sections from 6- and 20-month-old 3xTg-AD mice were doubleabeled with M71/3 and anti-tau antibodies. These ages were selected because tau accumulation in the somatodendritic compartment is the earliest sign of tau pathology in the 3xTg-AD mice, which is first apparent by 6 months of age. By 20 months, the tangle pathology is quite advanced and apparent with different silver stains and also immunoreactive with several phosphospecific tau antibodies (e.g., AT8, PHF-1) (25). Our analysis indicates that there is some but not complete co-localization between the M71/3 and HT7 immunoreactivity (Fig. 4, A–C). In contrast we found scant evidence of M71/3 and AT8 co-localization in 20-month-old mice (Fig. 4, D–F). These results indicate that Aβ oligomers co-localize with HT7-positive somatodendritic tau deposits. The co-localization of Aβ oligomers with early tau pathology may be fortuitous or given the growing appreciation that Aβ can induce the tau pathology, it is tempting to speculate that Aβ oligomers acting either intraneuronally or extraneuronally at the synaptic surface may contribute to the development of the tau pathology.

Anti-oligomeric Aβ Antibody Clears Tau Pathology as Well as Aβ Pathology—We previously showed that a single injection of an anti-Aβ antibody clears Aβ deposits from the brains of the 3xTg-AD mice, which consequently leads to the reduction in early tau pathology (6). As it is plausible that only certain Aβ species may also result in clearance of tau, we determined the effects of an Aβ oligomeric-specific antibody on the clearance of the Aβ and tau pathology. We injected 2 μg of antibody A11 into the hippocampus of 12-month-old hemizygous 3xTg-AD mice. This antibody was used because it is highly selective for oligomeric Aβ, whereas M71/3 does not discriminate between oligomers and fibrils. We found that 7 days after a single administration of antibody A11, the Aβ pathology was reduced in the areas surrounding the injection site (Fig. 5, A–C). Remarkably, the removal of Aβ oligomers led to the clearance of the early tau pathology in the somatodendritic compartment of CA1 neurons (c.f. Fig. 5D–F). No clearance of Aβ or tau pathology was observed in the un.injected, contralateral hippocampus that served as an internal control (Fig. 5, A and D). We also injected 3xTg-AD mice with phosphate-buffered saline or an isotype control antibody and found that neither of these treatments had any effects on the Aβ and tau pathology (data not shown). These data strongly suggest that Aβ oligomers may be link between Aβ and tau pathology. Thus, besides impairing LTP (18, 22) and cognitive functions (23), Aβ oligomers can interact with tau pathology.
Aβ Oligomers in the Transgenic Brain

DISCUSSION

One of the earliest clinical manifestations that occurs in AD is a profound memory loss. Recently, it has been proposed that this initial memory loss may be caused by synaptic failure, which may be a consequence of the accumulation of soluble Aβ oligomers (35, 36). This hypothesis is further strengthened by the isolation of oligomers from AD brains (29, 32). In particular, McLean and colleagues (32) showed that this soluble form of Aβ greatly correlates with the severity of the disease. Aβ oligomers isolated from AD brains, human cerebrospinal fluid, or cell lines are resistant to common solvents such as SDS (28, 29, 32, 33, 37). In this study, we showed that the 3xTg-AD mice develop SDS-resistant oligomers in a progressive manner, suggesting that these structures are fairly stable. This is notable considering that recently it has been proposed that Aβ oligomers may not just be a transient stage to fibrils, but they may be metastable structures that interfere with neuronal function (34).

One of the major findings of this work was that Aβ oligomerization starts intraneuronally. These data are consistent with previous data showing that Aβ oligomerization also begins intracellularly in primary human neurons and in other neuronal cell lines and in transgenic mice (27, 28). Recent studies have shown that intraneuronal Aβ plays a pathophysiological role in the progression of the disease (reviewed in Ref. 12). In particular, we previously showed that the 3xTg-AD mice have a selective loss of α7 nicotine acetylcholine receptors (α7nAChRs) restricted to brain regions that accumulate intraneuronal Aβ (38). Moreover, we showed that 6-month-old 3xTg-AD mice have profound LTP and cognitive deficits caused by the loss of α7nAChRs and the LTP and cognitive deficits in the 3xTg-AD mice. On the other hand, there is also evidence that Aβ oligomers can act as extracellular ligands for synapses (29, 40). These two views are not mutually exclusive. Considering the potency of Aβ oligomers, it is possible that these structures are bioactive within the cell, interfering with cell function, and also, once in the extracellular space (Fig. 2), they can act as a ligand and interfere with general neuronal networking. Moreover, we cannot exclude the possibility that some Aβ oligomers may also form extracellularly and further contribute to Aβ toxicity and fibril formation. Additional studies will be necessary to determine the extent of the contribution of Aβ oligomers to the loss of α7nAChRs and the LTP and cognitive impairments in the 3xTg-AD mice. Nevertheless, the data presented here, along with other studies showing that Aβ oligomers impair LTP and cognitive functions (18, 19, 22, 23), strongly argue in favor of this hypothesis.

We and others presented data to show that Aβ and tau pathologies interact in vivo (4–6). In particular, we have shown that anti-Aβ antibodies clear both intracellular and extracellular Aβ deposits and lead to the subsequent clearance of the early tau pathology (6). In this study, we showed that a single intrahippocampal injection of the oligomeric-specific antibody A11 reduces intracellular Aβ deposits. In this regard, we have previously shown that intraneuronal Aβ deposits can also be removed by other anti-Aβ antibodies (6, 39). The mechanism underlying the removal of intraneuronal Aβ seems to be because of a dynamic relationship between intraneuronal and extracellular pools of Aβ (41). Notably, the injection of the anti-oligomeric Aβ antibody A11, leads to the reduction of tau pathology as well as Aβ pathology. These data strongly suggest that Aβ oligomers may represent a link between Aβ and tau pathology.

It has recently been shown that Aβ immunotherapy neutralizes Aβ oligomers, thus rescuing the oligomer-induced LTP deficit (24). Furthermore, Walsh and colleagues (42) showed that the oligomer-induced LTP deficit can be rescued by small molecules that block Aβ oligomerization. In consideration of these data and the findings presented here, it is plausible to speculate that blocking Aβ oligomerization is a valid therapeutic target to lessen or halt AD neurodegeneration, as preventing Aβ oligomer formation can rescue the LTP and behavioral impairments and the early tau pathology. This last point is very important as tau pathology is sufficient to cause neurodegeneration in humans;


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to remove/halt the progression of tau pathology as well as Aβ pathology.

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