The Oligomerization of Amyloid β-Protein Begins Intracellularly in Cells Derived from Human Brain†

Dominic M. Walsh,‡ Bertrand P. Tseng,‡ Russell E. Rydel,§ Marcia B. Podlisny,‡ and Dennis J. Selkoe*,‡

Department of Neurology and Program in Neuroscience, Harvard Medical School and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, Massachusetts 02115, and Elan Pharmaceuticals, Inc., 800 Gateway Boulevard, San Francisco, California 94080

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ABSTRACT: The progressive aggregation and deposition of amyloid β-protein (Aβ) in brain regions subserving memory and cognition is an early and invariant feature of Alzheimer's disease, the most common cause of cognitive failure in aged humans. Inhibiting Aβ aggregation is therapeutically attractive because this process is believed to be an exclusively pathological event. Whereas many studies have examined the aggregation of synthetic Aβ peptides under nonphysiological conditions and concentrations, we have detected and characterized the oligomerization of naturally secreted Aβ at nanomolar levels in cultures of APP-expressing CHO cells [Podlisny, M. B., Ostaszewski, B. L., Squazzo, S. L., Koo, E. H., Rydell, R. E., Teplow, D. B., and Selkoe, D. J. (1995) *J. Biol. Chem. 270*, 9564−9570 (*1*); Podlisny, M. B., Walsh, D. M., Amarante, P., Ostaszewski, B. L., Stimson, E. R., Maggio, J. E., Teplow, D. B., and Selkoe, D. J. (1998) *Biochemistry 37*, 3602−3611 (*2*)]. To determine whether similar species occur in vivo, we probed samples of human cerebrospinal fluid (CSF) and detected SDS-stable dimers of Aβ in some subjects. Incubation of CSF or of CHO conditioned medium at 37 °C did not lead to new oligomer formation. This inability to induce oligomers extracellularly as well as the detection of oligomers in cell medium very early during the course of pulse−chase experiments suggested that natural Aβ oligomers might first form intracellularly. We therefore searched for and detected intracellular Aβ oligomers, principally dimers, in primary human neurons and in neuronal and nonneural cell lines. These dimers arose intracellularly rather than being derived from the medium by reuptake. The dimers were particularly detectable in neural cells: the ratio of intracellular to extracellular oligomers was much higher in brain-derived than nonbrain cells. We conclude that the pathogenically critical process of Aβ oligomerization begins intraneuronally.

Alzheimer's disease (AD) is the most common cause of progressive cognitive impairment in older humans (*3*). Substantial genetic, neuropathological, and animal modeling data indicate that amyloid β-protein (Aβ) plays an initiating role in a complex cascade which culminates in clinical dementia (*4*). A central question about AD pathogenesis is the mechanism by which soluble Aβ monomers produced constitutively by brain cells are converted to innumerable, plaque-associated extracellular fibrils. Understanding how and where this process of Aβ aggregation begins and developing systems to examine and inhibit early Aβ oligomerization under physiologically relevant conditions are key goals in the effort to design effective anti-aggregating drugs for treating or preventing AD.

Until recently, it was generally hypothesized that Aβ had to be assembled into highly insoluble extracellular amyloid fibrils to exert its cytotoxic effects (*5*−*8*). However, there is an emerging consensus that prefibrillar, diffusible assemblies of Aβ are also deleterious, as suggested by studies conducted in vitro (*9*−*11*) and in vivo (*12, 13*). Thus, therapeutic strategies involving inhibition of fibrillogenesis or dissociation of preexisting fibrils could be counterproductive if they elevate toxic prefibrillar moieties. Moreover, very recent studies suggest that intraneuronal accumulation of Aβ peptides of unknown assembly state may precede the detection of extracellular amyloid plaques, both in humans and in APP transgenic mice, and may be associated with neurodegeneration (*13, 14*). Evidence linking intracellular and extracellular Aβ comes from the finding in cell culture that exogenous fibrillar Aβ can induce intracellular accumulation of endogenous Aβ (*15*). Hence, before effective intervention can be contemplated, it is important that a detailed understanding of the various types of Aβ assemblies and their sites of origin be obtained.

Several laboratories have reported that Aβ can exist as stable, SDS-resistant oligomers in normal and AD brain (*16*−*19*) and in the conditioned media of APP-transfected CHO cells (*1, 2*). The latter species are 8−16 kDa Aβ-immunoreactive proteins confirmed to be bona fide Aβ oligomers by N-terminal radiosequencing and precipitation by C-terminal specific Aβ antibodies (*1, 2*). Their pathogenic relevance is suggested by the finding that AD-causing

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* To whom correspondence should be addressed at the Center for Neurologic Diseases, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur (Room 730), Boston, MA 02115-5716. Ph: 617-525-5200. Fax: 617-525-5252. E-mail: selkoe@cnd.bwh.harvard.edu.

‡ Harvard Medical School and Center for Neurologic Diseases, Brigham and Women's Hospital.

§ Elan Pharmaceuticals, Inc.

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mutations in APP or presenilins 1 or 2, which elevate Aβ42 production, significantly increase the amount of these oligomers in CHO media (*20*). However, it has not been clear where such Aβ oligomers originate, i.e., whether they form intracellularly or only after secretion of monomer. In this regard, formic acid extraction of cultured human NT2N neuronal cells results in the solubilization of a pool of otherwise undetectable intracellular Aβ (*21*). This formic acid soluble pool is exceptionally stable and accumulates with time in culture (*21*). Formic acid treatment has been shown to disrupt Aβ binding to carrier proteins in human plasma (*22, 23*). Therefore, the pool released by formic acid treatment of the NT2N neurons could either represent soluble Aβ monomers bound to carrier protein(s) or an aggregated form of intraneuronal Aβ that requires the use of formic acid to solubilize it and render it detectable by ELISA. Whether the detergent-insoluble intraneuronal species released by formic acid extraction actually represent oligomeric Aβ and how they relate to extracellular Aβ oligomers are unclear.

Here, we searched for naturally occurring Aβ oligomers originating from human brain by probing cerebrospinal fluid with a sensitive immunochemical method and found small amounts of SDS-stable dimers in some CSF samples. These had similar properties, including size and immunoreactivity, as Aβ dimers in CHO medium previously confirmed by radiosequencing (*1, 2*). Additional oligomer formation could not be induced by incubation at 37 °C of CSF or CHO medium. Moreover, pulse−chase experiments on CHO cells revealed initial detection of Aβ oligomers in medium very early during the chase. In view of these results, we sought to determine whether Aβ oligomers first arise intracellularly. Lysates from primary human neurons and two human neural cell lines contained an Aβ-immunoreactive species migrating at ~8 kDa (a putative dimer) that comigrated with a similar band in the lysates of APP-transfected CHO cells bearing confirmed dimers in their medium. Importantly, the intracellular:extracellular ratio of oligomers was substantially higher in neural cells than in CHO cells. Thus, in contrast to the common assumption that Aβ aggregation is initiated in the extracellular space after secretion of the monomer, our results suggest that Aβ oligomerization begins inside neurons.

EXPERIMENTAL PROCEDURES

APP-Transfected Cell Lines. Chinese hamster ovary (CHO) cells stably transfected with a cDNA encoding the 751 residue form of β-amyloid precursor protein (APP 751, the major APP isoform that peripheral cells express) containing the Val717Phe familial AD mutation (referred to as 7PA2 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone), as previously described (*1*). Human neuroglioma HS683 and neuroblastoma SK-N-SH cell lines, each stably transfected with APP695 cDNA (the major APP isoform that neural cells express) (referred to as SK_{695} and HS_{695} cells), were cultured similarly (*24, 25*).

Primary Human Cortical Cultures. Primary fetal human cortical cultures were prepared using a modification of a procedure described previously (*26*). Cortical tissue was dissociated by trituration followed by incubation in a trypsin/EDTA solution [0.05% trypsin + 0.53 mM EDTA in Hank's balanced salt solution (HBSS, GibcoBRL)] for 20 min at 37 °C. The trypsin was then inactivated by resuspending the cells in serum-containing medium: Minimum Essential Medium with Earle's salts (MEM) (GibcoBRL) containing 1% glucose, 1 mM sodium pyruvate, 1 mM glutamine, and 10% fetal bovine serum. Cells were then pelleted by centrifugation and resuspended in a chemically defined medium: MEM containing B27 supplement (MEM/B27) (GibcoBRL) in place of FBS. Polyethylenimine-coated 35 mm, 6-well plates were rinsed once with MEM/FBS and then seeded at 4×10^6 cells per well in 2 mL of MEM/B27. Cultures were maintained in a water-saturated incubator with an atmosphere of 95% air/5% CO_2 at 37 °C, and the medium was changed twice weekly.

Whole Cell Lysates. Lysates of naive and APP-transfected cell lines were prepared essentially as described (*27*). Cells were grown in 10 cm^2 dishes until nearly confluent, washed twice, transferred into serum-free medium, and incubated for ~16 h. Conditioned media were removed, and the cells were washed with ice-cold PBS (10 mL/10 cm^2 dish ×2) and lysed with 1% NP-40 in 50 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 2 mM EDTA, 50 μg/mL leupeptin, 950 μg/mL aprotinin, 20 μg/mL pepstatin A, and 120 μg/mL PEFA block. To ensure that the Aβ signal detected was intracellular in origin and not due to extracellular Aβ adhering to the plasma membrane, some cultures were placed on ice and treated with trypsin (500 μg/dish) for 15 min. The trypsin was inhibited by the addition of serum-containing medium, and then the cells were treated as above. For examination of intracellular Aβ by our immunoprecipitation/Western blot method, immunoprecipitates of combined lysates from seven 10 cm^2 dishes were loaded in each gel lane.

Neuronal cultures were maintained for 3−4 weeks, washed twice with PBS, and then incubated for ~22 h in 1.5 mL of MEM/B27. Conditioned media were removed and cleared by centrifugation, and the cells were washed twice with ice-cold PBS and lysed as above, using 250 μL of buffer/well. Immunoprecipitated material from 5 to 7, 6-well plates was loaded in each gel lane. Following collection, cell lysates and conditioned media were frozen on dry ice/ethanol and stored at −80 °C (<2 weeks) pending analysis.

Cerebrospinal Fluid. Samples (1.0−9.0 mL) of human cerebrospinal fluid (CSF) from living patients were obtained from two sources. First, discarded samples from patients undergoing routine lumbar puncture in the Department of Neurology at Brigham and Women's Hospital were stored at 4 °C immediately after collection; within 4−24 h, they were cleared by low-speed centrifugation, divided into 1−2 mL aliquots, frozen, and stored at −80 °C until used (≤3 months). Second, five samples from our laboratory's CSF archive which had originally been obtained from the AD Research Center at Rush Presbyterian St. Lukes Hospital (Chicago) had been stored at −80 °C for ~9 years prior to analysis.

Antibodies and Enzyme-Linked Immunosorbant Assay for Aβ. The antibodies used in this study are listed in Table 1.

Preclearing with the anti-APP antibodies B5 and C7 was found to substantially enhance the ability to detect intracellular Aβ. Enzyme-linked immunoassays (ELISA) for Aβ1-total (all Aβ species beginning at Asp1) and Aβ1−42 were performed as described (*28*). Full-length APP and APPs-α are not detected by either of these assays (*32*).

Table 1

name	monoclonal/ polyclonal	epitope	source and reference
3D6	M	Aβ1−5	Elan Pharmaceuticals (*28*)
6C6	M	Aβ1−16	Elan Pharmaceuticals (*28*)
6E10	M	Aβ1−17	Senetek Inc. (*29*)
4G8	M	Aβ17−24	Senetek Inc. (*29*)
2G3	M	Aβ31−40 (40 specific)	Elan Pharmaceuticals (*28*)
21F12	M	Aβ33−42 (42 specific)	Elan Pharmaceuticals (*28*)
R1280	P	raised to Aβ1−40	Selkoe Lab (*30*)
R1282	P	raised to Aβ1−40	Selkoe Lab (*30*)
B5	P	raised to APP444−592	Elan Pharmaceuticals (*31*)
C7	P	raised to APP676−695	Selkoe Lab (*30*)

Immunoprecipitation and Gel Fluorography. Nearly confluent (95−100%) 10 cm^2 dishes of 7PA2 cells and their corresponding untransfected parental CHO cell line were starved of methionine for 30 min and then labeled with 750 μCi of [^{35}S]methionine, and their media were harvested and immunoprecipitated (*1*). Following electrophoresis on 16% Tricine gels, bands were visualized by gel fluorography. For experiments examining the ability of Aβ oligomers to form in conditioned media in the absence of cells, 7PA2 cells were pulsed with 1 mCi of [^{35}S]methionine for 2 h. The labeled medium was harvested, cleared of cells, and incubated either at 4 °C or at 37 °C for 15 h in the presence or absence of CHO cells (plus or minus protease inhibitors as indicated) and then immunoprecipitated with R1282. As positive controls, 7PA2 cells were labeled for 17 h and their conditioned media immunoprecipitated as above.

For pulse−chase experiments designed to determine the earliest time point at which oligomers could be detected in conditioned medium, three 10 cm^2 dishes of nearly confluent 7PA2 cells were each pulsed with 2 mCi of [^{35}S]methionine for 20 min and chased in 20 min intervals. At each time point, the media from the three dishes were combined and used for immunoprecipitation with R1280, and immunoreactive species were detected by phosphorimaging using a model 425E phosphoimager (Molecular Dynamics, Sunnyvale, CA).

IP/Western Blot Analysis. To visualize steady-state levels of Aβ in human CSF and in cultures that were not radiolabeled, we devised an immunoprecipitation/Western blot (ip/wb) protocol that allowed the highly sensitive detection of unlabeled Aβ species. Analysis of conditioned media by ELISA and ip/wb revealed that our ip/wb protocol can readily detect as little as 200 pg of endogenously secreted Aβ (not shown). Samples were immunoprecipitated so as to avoid reconstitution procedures which might alter the assembly form or recovery of Aβ. Following immunoprecipitation (described above), samples were electrophoresed on 16% Tricine gels and transferred onto 0.2 μM nitrocellulose membranes at 400 mA for 2 h. Filters were boiled for 10 min in PBS (*33*) and blocked overnight at 4 °C with 5% fat-free milk in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.05% Tween 20 (TBS-T). After washing the membranes in TBS-T, monoclonal antibody 6E10 or a combination of monoclonals 4G8 and 6C6 (each at 1 μg/mL) were used to probe the blots. Bound antibody was visualized using horseradish peroxidase-conjugated anti-mouse Ig (at 1:40 000) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and ECL+ detection (Amersham Pharmacia Biotech, Arlington Heights, IL).

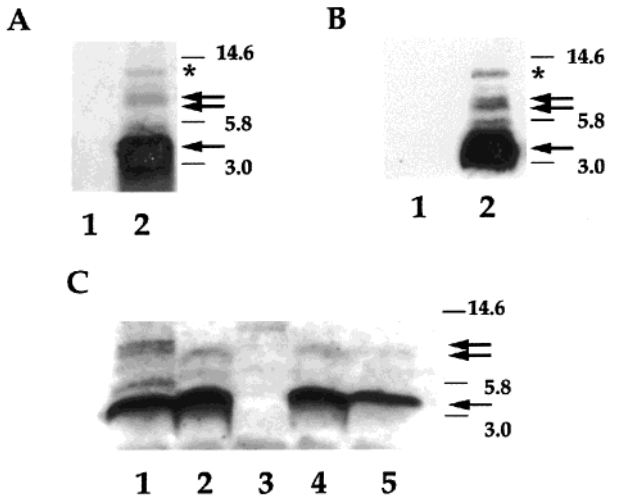

FIGURE 1: Detection of SDS-stable oligomers of Aβ in conditioned media and human CSF. (A) 7PA2 cells were grown to near-confluence in a 10 cm^2 dish and labeled for 16 h with [^{35}S]-methionine as per Experimental Procedures. Conditioned medium immunoprecipitated with preimmune serum (lane 1) or R1282 (lane 2) was examined by phosphorimaging. Arrow indicates monomeric Aβ, double arrow indicates dimeric Aβ (which frequently appears as a doublet), and asterisk indicates trimeric Aβ. (B) 7PA2 cells were grown to near-confluence in a 10 cm^2 dish and conditioned for 16 h as described in (A) but without label. Conditioned media immunoprecipitated with preimmune serum (lane 1) or R1282 (lane 2) were Western blotted with the monoclonal antibody 6E10 (to Aβ1−17). Arrows and asterisk as in (A). (C) Aliquots of CSF (lanes 2 and 3: 1.5 mL; lane 4: 1.0 mL; lane 5: 0.5 mL) from an 83 year old male with probable multi-infarct dementia were immunoprecipitated with either R1280 (lanes 2, 4, and 5) or preimmune antiserum (lane 3) and Western blotted with a combination of monoclonal antibodies 6C6 and 4G8. As a positive control, 7PA2 cell conditioned medium (0.5 mL) was immunoprecipitated identically with R1280 (lane 1). Arrows as in (A) and (B).

RESULTS

SDS-Stable Oligomers of Aβ Are Present in the Medium of APP-Expressing CHO Cells and in Some Human CSF Samples. We previously reported that CHO cells stably transfected with an APP$_{751}$ cDNA bearing the Val717Phe FAD mutation (7PA2 cells) contain Aβ species in their medium that migrate on SDS−PAGE at ~4, 6, 8 (a doublet), and 12 kDa (*1*) (Figure 1A). By comparison to prestained markers (MWs of 3045, 5820, 14 585, 20 280, 29 025, and 46 060), we estimated the apparent molecular weight of each Aβ species. Based on analysis of eight different Western blots, each of which contained at least one lane of these standards and two lanes of immunoprecipitated 7PA2 conditioned medium, we obtained average molecular weights of 4319 (~4 kDa), 5919 (~6 kDa), 7575 & 8255 (~8 kDa doublet), and 11 047 (~12 kDa) for the respective Aβ species. Several criteria, including radiosequencing and immuoprecipitation with C-terminal specific anti-Aβ antibodies, clearly established that the 8 and 12 kDa bands represent SDS-stable Aβ oligomers, namely, dimers and trimers (*1, 2*). To demonstrate such oligomers, we have generally employed metabolic labeling of confluent cultures and subsequent immunoprecipitation of the labeled medium with highly specific Aβ antibodies followed by gel fluorog-

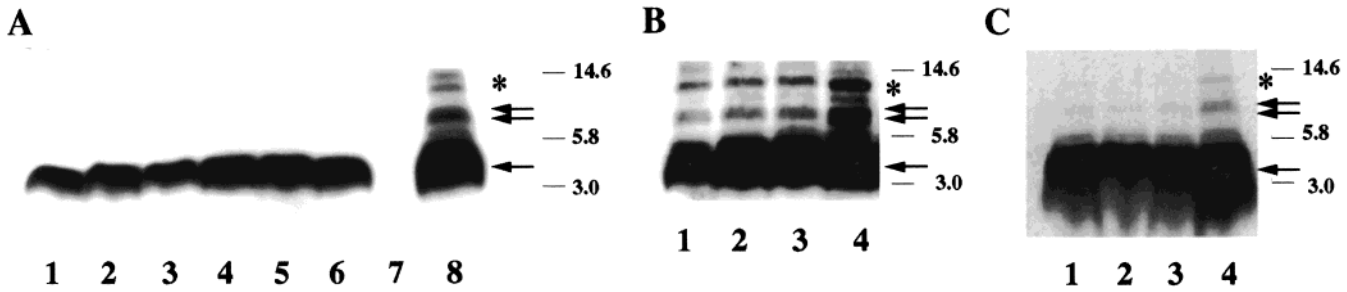

FIGURE 2: Aβ in extracellular fluids cannot be induced to oligomerize by incubation in vitro at 37 °C. (A) Effect of incubating human CSF (2.1 mL per aliquot) at 37 °C with and without protease inhibitors (PI) was investigated. Lanes 1−4: CSF from an 82 year old patient with normal pressure hydrocephalus was incubated at 37 °C without (lane 1) or with (lane 3) PI for 14 h, or at 4 °C without (lane 2) or with (lane 4) PI. Lanes 5 and 6: CSF from a 27 year old patient with multiple sclerosis was incubated at 37 °C (lane 5) or 4 °C (lane 6) with PI. Lane 8: Unincubated conditioned medium from 7PA2 cells (2 mL) was used as a positive control. Lane 7 is empty. All samples were immunoprecipitated with R1280 and Western blotted with 6E10. (B) Three 10 cm^2 dishes of confluent 7PA2 cells were allowed to condition serum-free medium for 2 h. The media were collected, cleared of cells, pooled, and divided into 3 equal aliquots. Aliquots were incubated in vitro at 37 °C for 15 h without (lane 1) or with (lane 2) PI, or incubated at 4 °C with PI (lane 3). Medium from a 10 cm^2 dish of confluent 7PA2 cells conditioned for 17 h was used as a positive control (lane 4). All samples were examined by ip/wb as in (A). (C) Three 10 cm^2 dishes of confluent 7PA2 cells were labeled with [^{35}S]methionine for 2 h, and the conditioned medium was removed, cleared of cells, and incubated for 15 h at 37 °C with (lane 1) or without (lane 2) PI or at 4 °C (lane 3). 7PA2 medium labeled for 17 h served as a positive control (lane 4). All samples were immunoprecipitated with R1282 and examined by phosphorimaging. In A, B, and C, single arrow indicates monomeric Aβ, double arrow indicates dimeric Aβ (which frequently appears as a doublet), and asterisk indicates trimeric Aβ.

raphy (Figure 1A). To detect steady-state oligomer levels in samples such as human CSF that cannot be radiolabeled, we developed a sensitive immunoprecipitation/Western blotting (ip/wb) protocol that employed our high-titer anti-Aβ antibodies R1280 or R1282 for ip and monoclonal antibody 6E10 or a combination of monoclonal antibodies 4G8 and 6C6 for Western blotting. Comparison of Figure 1A and Figure 1B reveals that ip/wb (B) yielded results qualitatively indistinguishable from but quantitatively more robust than those obtained by autoradiography of R1282 immunoprecipitates (A). To clearly visualize radiolabeled Aβ oligomers by phosphorimaging or gel fluorography (as in Figure 1A), phosphor screens or autoradiographic film typically needed to be exposed to the gels for 7−10 days, whereas our ip/wb technique (Figure 1B) yields results in ~8 h. It should be noted that, depending on the antibody used for Western blotting, the p3 protein (i.e., Aβ17−40/42, the product of the α- and γ-secretase cleavages of APP) may not be detected; e.g., antibody 6E10 raised to Aβ1−17 cannot detect p3 (Figure 1B).

To search for the occurrence of similar SDS-stable oligomers of naturally secreted Aβ arising from the brain in vivo, human CSF samples were analyzed using this ip/wb protocol. Among 56 human CSF samples examined, Aβ immunoreactive bands migrating higher than 4 kDa similar to those present in 7PA2 conditioned medium were detected in 15 samples (Figure 1C). These bands were Aβ-specific, as they were not visualized when CSF was precipitated with preimmune serum (Figure 1C, lane 3) or when the primary antibody used for Western blotting was omitted (data not shown). The ~7.5 kDa Aβ species observed in CSF did not comigrate precisely with the dimeric (~8 kDa) bands in 7PA2 conditioned media (Figure 1C, lanes 1 and 2). This slight difference may be due to the documented N-terminal heterogeneity between CSF Aβ species (*34*) and those secreted by 7PA2 cells (*1*). For a given CSF sample, detectability of oligomers was dependent on the volume of CSF used; e.g., the ~7.5 kDa oligomer was readily detected when 1.5 mL of the sample was probed (Figure 1C, lane 2), but was less obvious when 1 mL (lane 4) was used and essentially undetectable when 0.5 mL (lane 5) was used. Most of the 56 available CSF samples we probed in this way showed no clear SDS-stable oligomeric species; however, many of the samples were too limited in volume (<1.5 mL) to allow optimal ip/wb analysis. No relationship between the presence of Aβ oligomers and age or neurological diagnosis was observed.

Aβ in Extracellular Fluids Undergoes No Detectable Induction of Oligomers during Incubation at 37 °C. CSF samples from six subjects who did not show detectable SDS-stable Aβ oligomers by ip/wb were used to determine whether Aβ oligomer formation could be induced by incubation at 37 °C. Each sample was thawed and divided into 4 aliquots of ≥2.1 mL each. A cocktail of protease inhibitors (see Experimental Procedures) was added to 2 of the 4 aliquots from each subject, and aliquots with and without the inhibitors were then incubated for 14 h at either 4 or 37 °C. Samples were then placed on ice, an aliquot was taken for Aβ ELISA, and the remainder was immunoprecipitated with the anti-Aβ antibody R1280 and blotted with 6E10. In all six subjects, we detected no Aβ oligomers either before or after the 37 °C incubation (with or without protease inhibitors), despite the presence of abundant monomers (Figure 2A). These results indicate that the Aβ monomers present in human CSF do not readily oligomerize under the conditions used. Due to limited volumes of the appropriate samples, we could not test for the enhancement of oligomers in those CSF samples which already had detectable SDS-stable oligomers.

For this reason, we examined conditioned media from 7PA2 cells to determine whether oligomerization of naturally secreted Aβ could be induced extracellularly in a cell line that spontaneously displays oligomers of Aβ in its medium. Confluent 7PA2 cultures were conditioned for 2 h, and the media were collected and cleared of cells. The media were incubated at either 4 or 37 °C for 15 h in the presence or absence of untransfected CHO cells and with or without protease inhibitors. The incubated media were then placed on ice, sampled for ELISA, and examined by immunoprecipitation with R1282 and Western blotting with 6E10.

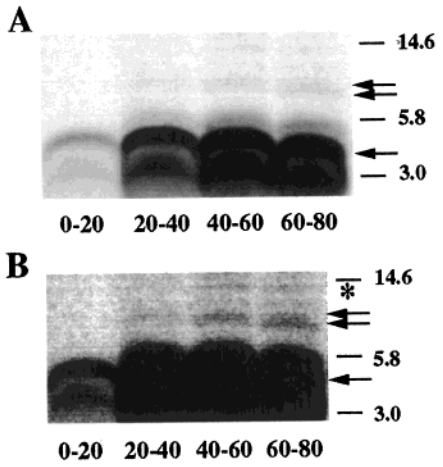

FIGURE 3: Appearance of dimeric (~8 kDa) Aβ early in the course of pulse−chase experiments. (A) 7PA2 cells (three 10 cm^2 dishes) were pulsed with [^{35}S]methionine for 20 min as per Experimental Procedures and chased in 20 min intervals. Samples were examined with R1280 immunoprecipitation and phosphorimaging. Arrow indicates monomeric Aβ; double arrow indicates dimeric Aβ. (B) The same phosphorimage as in (A) was overexposed to allow better visualization of the oligomeric bands. A faint ~12 kDa trimeric band can be seen (*).

Dimeric (~8 kDa) and trimeric (~12 kDa) Aβ were detectable in this 2 h conditioned sample (Figure 2B, lane 3), but further incubation at 37 °C either in the absence (lane 1) or in the presence (lane 2) of protease inhibitors did not alter the intensity of these bands. Next, induction experiments using conditioned media of [^{35}S]methionine-labeled cells were performed identically and examined by R1282 immunoprecipitation and gel fluorography (Figure 2C). Phosphorimaging yielded similar results to those obtained by ip/wb; i.e., the levels of oligomers were unchanged by incubation at 37 °C, but the oligomeric bands detected by this method were less intense than those visualized by ip/wb (compare Figure 2B and 2C). Incubation of the media in the presence of CHO cells caused a marked reduction in the ELISA-measured Aβ concentration and in the levels of the 4 kDa monomer band on gels, but did not alter the oligomeric bands (data not shown), as expected from published evidence that CHO cells secrete proteases which can efficiently degrade monomeric but not oligomeric Aβ (*35*, *36*).

The inability to induce Aβ oligomer formation in both human CSF and isolated Aβ-rich conditioned medium raised the possibility that Aβ oligomers might not arise in the extracellular fluid but rather intracellularly. To obtain further evidence for this hypothesis, we compared the temporal course of appearance of various newly secreted Aβ species in the conditioned media of 7PA2 cells. Pulse−chase analysis revealed that very small amounts of SDS-stable ~8 kDa dimers could be observed in the conditioned media of 7PA2 cells as early as 20 min after the secreted monomer (4 kDa) was first detectable (Figure 3A and 3B, compare 0−20 min vs 20−40 min chase periods). It should be noted that even after 15 h of continuous metabolic labeling, oligomers represent a small percentage of the total Aβ signal detected by gel fluorography in 7PA2 medium (Figures 1A, lane 2, and 2C, lane 4) (*2*). Therefore, the apparent lack of dimers in the first 20 min chase period (when the monomer is quite faint) probably signifies that the amount present is below the detection limit. The appearance of the labeled dimers during the second 20 min chase indicates that Aβ oligomers are either secreted as such by the cells or else form in the medium very soon after secretion of the labeled monomer.

Detection of SDS-Stable Oligomers of Aβ Intracellularly in Neurons and Nonneuronal Cells. To address directly whether Aβ oligomerization begins intracellularly, total cell lysates were probed by our sensitive ip/wb method. Lysates prepared from seven 10 cm^2 dishes of confluent 7PA2 cells or the untransfected CHO parental cells were extensively precleared with a combination of a polyclonal antibody (C7) recognizing full-length APP and its C-terminal fragments and a polyclonal antibody (B5) to APPs-α and -β, to deplete any APP forms that could compete with Aβ for immunoprecipitation. The precleared lysates were then precipitated with either preimmune serum, R1280 antiserum, or 2G3 or 21F12 monoclonal antibodies. Lysates from fourteen 10 cm^2 dishes of confluent 7PA2 cells were used for each immunoprecipitation with C-terminal specific monoclonals. All immunoprecipitates were then subjected to Western blotting with 6E10 (Figure 4A). This revealed a pattern similar to that seen in conditioned medium (Figures 1−3): a robust 4 kDa band, a band at ~5.5 kDa, and a doublet at ~8 kDa (Figure 4A, lanes 2, 5, and 6). In lysates immunoprecipitated with 2G3 or 21F12, a trimer band (~12 kDa) was also evident (Figure 4A, asterisk). This band was not clearly evident in R1280 immunoprecipitates because R1280 also recognizes C-terminal fragments of APP which contain all or part of the Aβ sequence and which migrate in this region and obscure detection of trimeric Aβ. No such bands were detected when preimmune serum was used (lane 4) or when untransfected CHO cell lysates were immunoprecipitated with R1280 (lane 3), demonstrating the specificity of the bands. Moreover, the preclearing step with APP antibodies C7 and B5 yielded no bands at ~4, 6, or 8 kDa (lane 1). Brief trypsinization of the cells (see Experimental Procedures) to remove residual Aβ adherent to the cell surface prior to lysis did not change these results (data not shown). These findings, taken together with our observation that extracellular Aβ cannot be induced to form oligomers (Figure 2), strongly suggest that SDS-stable oligomers of Aβ (particularly dimers) are initially formed intracellularly, and at least a portion of them is then secreted.

To determine if such intracellular Aβ oligomers also occur in cells of brain origin, we probed SK_{695} and HS_{695} human cell lines (see Experimental Procedures) as well as primary human cortical cultures for the presence of these species. Aβ-specific bands migrating as monomers and dimers were detected in SK_{695} cell lysates (Figure 4B, lane 5) but not in those of the untransfected parent SK-N-SH cell line (lane 4). The bands detected in SK_{695} lysates (lane 5) appeared similar to those found in the 7PA2 cell lysates (lane 6), but the dimer:monomer ratio was much higher in the former, and there appeared to be only a single dimer band in the SK_{695} cells, whereas a tightly spaced doublet at ~8 kDa was apparent in the 7PA2 cells. No oligomeric bands were detectable in SK_{695} media when a total of 4 mL of conditioned media was probed (Figure 4B, lane 2), further supporting the concept that Aβ oligomers first appear intracellularly and may or may not be quantitatively secreted. This result also signifies that the intracellular oligomers (lane 5) could not have arisen from surface binding or uptake of oligomers from the medium (lane 2). We next examined

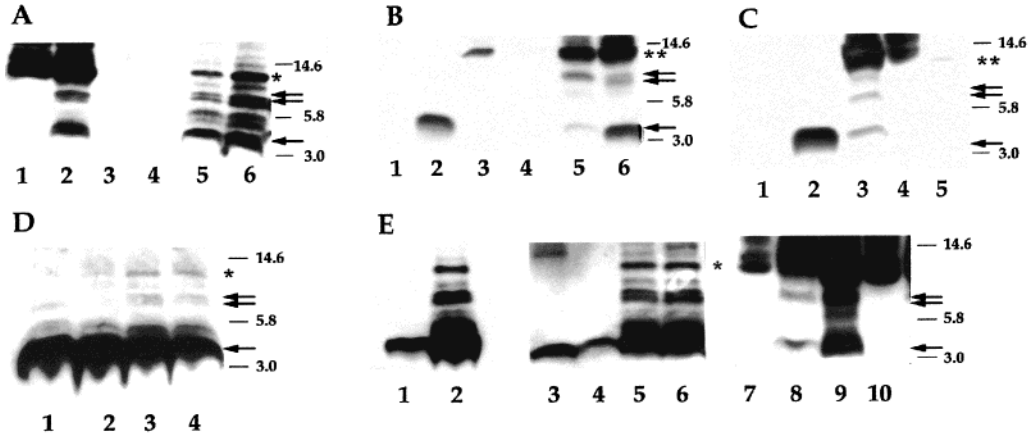

FIGURE 4: Detection of stable intracellular oligomers of Aβ in primary human cortical cultures and neural cell lines. (A) Chinese hamster ovary cells probed by ip/wb. Lysates of 7PA2 cells (lanes 1, 2, 4, 5, 6) or untransfected CHO cells (lane 3) were prepared as described and immunoprecipitated with R1280 (lane 2 and 3), or preimmune serum (lane 4) or monoclonal antibodies 21F12 (lane 5) or 2G3 (lane 6). Lane 1: material obtained by preclearing the lysate of lane 2 by immunoprecipitating with B5 and C7 prior to immunoprecipitation with R1280. Aβ-immunoreactive bands were detected by Western blotting with 6E10. Arrow: 4 kDa Aβ monomer; double arrow: ~8 kDa dimer. Trimeric Aβ (*) is readily detectable in 7PA2 lysates immunoprecipitated with 21F12 (lane 5) and 2G3 (lane 6); these antibodies do not recognize APP C-terminal fragments since they require free Aβ C-termini. (B) Human SK-N-SH neuroblastoma cells probed by ip/wb. Lanes 1 and 2: R1280 immunoprecipitates of conditioned media (4 mL each) of untransfected SK-N-SH cells (1) or SK_{695} cells (2). Lanes 4−6: R1280 precipitates of lysates of untransfected SK-N-SH cells (4), SK_{695} cells (5), or 7PA2 cells (6). Lane 3: material obtained by preclearing the same lysate shown in lane 5 by precipitating with B5 and C7 prior to precipitation with R1280. The ~10−12 kDa bands immunoprecipitated by B5/C7 are likely to be C-terminal fragments of APP which still contain all or part of the Aβ sequence. Since C7 is known to efficiently immunoprecipitate C-terminal fragments of APP, it is surprising that immunoprecipitation of the same lysate with R1280 produced a more robust band in this region. This may indicate that Aβ-immunoreactive species (e.g., trimers of Aβ) other than APP fragments are also immunoprecipiated by R1280. Arrow indicates monomeric Aβ, double arrow indicates dimeric Aβ, and double asterisk indicates ~10 and 12 kDa C-terminal fragments of APP. (C) Human HS683 glioblasoma cells probed by ip/wb. Lanes 1 and 2: R1280 immunoprecipitates of conditioned media (4 mL each) of untransfected HS683 cells (1) or HS_{695} cells (2). Lanes 3 and 5: lysates of HS_{695} cells (3) or untransfected HS683 cells (5) were precipitated with R1280. Lane 4: material obtained by preclearing the same lysate used in lane 3 by precipitating with B5 and C7 prior to precipitation with R1280. Arrow indicates monomeric Aβ, double arrow indicates dimeric Aβ, and double asterisk indicates ~10 and 12 kDa C-terminal fragments of APP. (D) Cell lines grown at varying cell densities were allowed to condition media for ~16 h to enable a comparison of oligomers using similar total Aβ concentrations. The number of cells per plate and the total Aβ concentration, respectively, were: lane 1: HS_{695}, 8.4×10^6 cells and 4.0 ng/mL; lane 2: SK_{695}, 8.8×10^7 cells and 3.8 ng/mL; lane 3: 7PA2, 4.6×10^6 cells and 6.5 ng/mL; lane 4: 7PA2, 2.8×10^6 cells and 3.8 ng/mL. Media were examined by ip/wb, as in (A−C). Arrow, monomeric Aβ; double arrow, dimeric Aβ; asterisk, trimeric Aβ. (E) Human primary cortical cultures probed by ip/wb. Lanes 1 and 2: R1280 immunoprecipitates of conditioned media of human cortical cultures (1) or 7PA2 cells (2). Lanes 3 and 5: conditioned media from cortical cultures (3) or 7PA2 cells (5) were precipitated with the Aβ40 C-terminal specific monoclonal, 2G3. Lanes 4 and 6: conditioned media from cortical cultures (4) or 7PA2 cells (6) were precipitated with the Aβ42 C-terminal specific monoclonal, 21F12. Lanes 8 and 9: lysates of cortical cultures (8) or 7PA2 cells (9) were precipitated with R1280. Lanes 7 and 10: material obtained by preclearing the same lysates used in lanes 8 and 9, respectively, by precipitating with B5 and C7 prior to precipitation with R1280. Arrow indicates monomeric Aβ, and double arrow indicates dimeric Aβ. Trimeric Aβ (*) is readily detectable in 7PA2 conditioned medium immunoprecipitated with 1280 (lane 2), 2G3 (lane 5), and 21F12 (lane 6). However, it is not possible to discern if trimers are present in lysates (lanes 8 and 9) due to the heavy staining of APP C-terminal fragments in the same region.

HS_{695} cell lysates (Figure 4C). The 4 kDa monomer was low in abundance, and an Aβ-specific band at ~7−8 kDa was almost equally abundant (Figure 4C, lane 3). A faint, Aβ-immunoreactive band was detected at ~9−10 kDa. As with the SK_{695} and 7PA2 cell lysates, the bands detected in the HS_{695} cells at ~4, 8, and 10 kDa were not present in lysates of untransfected cells (Figure 4C, lane 5) or lysates immunoprecipitated with APP antibodies B5 and C7 (lane 4), indicating that these bands are Aβ-specific. The medium (4 mL) of the HS_{695} cells contained the 4 kDa monomer but no detectable oligomers (Figure 4C, lane 2).

It was possible that the lack of oligomer detection in the media of the SK_{695} and HS_{695} cell lines resulted from their secreting much less Aβ than our 7PA2 cells. On average, these neural cells secrete about one-tenth as much total Aβ as do the 7PA2 cells, and even in the medium of 7PA2 cells, Aβ oligomers represent only a small percentage of the monomeric Aβ signal. Thus, the lack of quantifiable oligomers in the media of HS_{695} and SK_{695} cells could have been a function of the detection limit of our technique. Moreover, since Aβ aggregation is believed to be a concentration-dependent process (*37*), the lack of oligomers in HS_{695} and SK_{695} conditioned medium could well indicate that the Aβ levels in their media may not reach the critical concentration required for aggregation. To examine these issues, we conditioned the three cell lines at different cell densities, thus obtaining medium samples from each of the lines that had roughly equivalent concentrations of Aβ monomer (Figure 4D). The mean Aβ concentrations in these matched media samples determined by ELISA (based on three different dilutions each assayed in triplicate) were the following: HS_{695} cells, 4045 ± 384 pg/mL; SK_{695} cells, 3850 ± 331 pg/mL; 7PA2 cells (sample 1), 6537 ± 176 pg/mL; and 7PA2 cells (sample 2), 3788 ± 329 pg/mL. Aβ-immunoreactive bands at ~4 kDa were detectable in all these media samples (Figure 4D). As expected, bands at ~8 kDa (a doublet) and at 12 kDa were apparent in the conditioned media of 7PA2 cells (in both samples 1 and 2) (Figure 4D, lanes 3 and 4), but no clear oligomeric bands were detected in SK_{695} conditioned media (lane 2). A faint single band migrating at ~7 kDa was detected in the HS_{695} conditioned media (lane 1), suggesting that these cells are capable of producing some

SDS-stable oligomers. Because we had previously shown that the amount of oligomers in medium is greater in CHO cells having elevated Aβ42:$Aβ_{total}$ ratios (*1, 20*), the low abundance of oligomers in media conditioned by the neural cells could reflect in part the secretion of lower amounts of Aβ42. In this regard, ELISA revealed that Aβ1−42 accounted for ∼18% of the total Aβ species secreted by 7PA2 cells, whereas it accounted for only ∼12 and ∼3% of the Aβ secreted by SK_{695} and HS_{695} cells, respectively. We conclude that the APP-transfected neural cell lines SK_{695} and HS_{695} do not have appreciable amounts of SDS-stable oligomers of Aβ in their media and thus the oligomers detected in lysates from these cells could not derive from adventitiously cell-associated extracellular oligomers.

When lysates from primary human cortical cultures were examined, they were also found to contain low amounts of apparent Aβ dimers at ∼8 kDa (Figure 4E, lane 8), whereas media conditioned by these cells usually demonstrated only monomers (lanes 1, 3, and 4). As in the lysates of the three cell lines examined above, preimmune serum (not shown) or APP antibodies B5 plus C7 (lane 7) did not precipitate these bands, indicating that they are specific Aβ dimers and not C-terminal APP fragments. Again, the difference in oligomer:monomer ratios between the lysates and the media of the same cortical cultures (lanes 1, 3, and 4 vs lane 8) strongly supports the conclusion that the SDS-stable oligomers of Aβ occur intracellularly rather than being bound back to the external surface of the cells from the medium.

DISCUSSION

Although many studies support the hypothesis that progressive cerebral accumulation of Aβ plays a central role in AD pathogenesis, two key unresolved questions remain: (a) Can Aβ induce neuronal injury intracellularly or only after accumulating extracellularly? (b) Where do Aβ oligomers, increasingly believed to be the principal toxic form, first arise? Here we demonstrate that SDS-stable Aβ oligomers, particularly dimers, are present intracellularly in a variety of cell types, including primary human neurons. We provide several lines of evidence that stable oligomers of Aβ are initially formed intracellularly. First, oligomers cannot be induced to form extracellularly in 7PA2 conditioned medium or human CSF by prolonged incubation at 37 °C, despite the presence of substantial levels of Aβ monomers and the occurrence of oligomers in the medium and in some CSF samples. Second, Aβ oligomers, particularly dimers, are detectable in the medium very early in the course of pulse−chase experiments, suggesting they may have been secreted as such. Third, we directly demonstrate SDS-stable oligomers in cell lysates of several cell types using a sensitive and specific ip/wb method. Fourth, intracellular oligomers can be detected even in neural cells that have no detectable oligomers in their media, signifying that the oligomers must have arisen intracellularly.

To our knowledge, this report provides the first direct visualization of natural intracellular Aβ oligomers in primary neurons as well as neural and nonneural cell lines. An age-dependent increase in SDS-stable dimers in the CA1 region of human hippocampus has been reported to occur in late middle-age, prior to or without detection of amyloid plaques and neurofibrillary tangles (*18*). However, in this and similar studies of brain homogenates, it is unclear whether the oligomers detected occur inside cells and/or in the extracellular space (*17−19, 38*). Similarly, there is growing evidence that APP or presenilin transgenic mice undergo Aβ-induced neurotoxicity prior to the appearance of any extracellular Aβ plaques (*12, 13*) but whether this is due to intra- or extracellular Aβ oligomers (or both) is unknown. These observations of pre-plaque Aβ-related injury in vivo have focused special attention on the nature and site of origin of the first stable Aβ assembly form. In this regard, our studies strongly suggest that Aβ can already dimerize intraneuronally, that at least some of the dimers are highly stable (resisting disassembly in boiling SDS), and that the dimers may or may not be released from the cells. We are currently attempting to determine in which vesicular compartment the dimers arise; post Golgi-secretory vesicles and endosomes are two reasonable candidates.

If Aβ principally oligomerized post-secretion, one might have expected the monomeric Aβ present in conditioned medium to oligomerize during incubation at 37 °C. Because the assembly of proteins into oligomers is a concentration-dependent process (*37*), this test would only be valid if monomeric Aβ concentrations were at least equal to those of conditioned media in which Aβ oligomers are known to occur. In our experiments, 7PA2 cells were allowed to condition media for 2 h, at which time stable dimers and trimers were already clearly detectable, yet further incubation of the collected medium either in the presence or in the absence of untransfected CHO cells failed to significantly increase the amount of oligomers. Moreover, when CSF samples were incubated at 37 °C, Aβ oligomers could not be induced in vitro, despite our finding that Aβ dimers can be detected in human CSF. Additional evidence for the lack of a dynamic change in these naturally occurring, extracellular oligomers comes from our previous observations that: (a) the level of oligomers in 7PA2 CHO medium cannot be significantly decreased by the extracellular addition of Congo red (*2*), a compound which readily prevents oligomerization of synthetic Aβ peptides (*7*); and (b) substantial extracellular degradation of monomeric Aβ by secreted or cell-surface proteases does not lead to a coordinate decrease in the amount of stable oligomers in 7PA2 medium (*35, 36, 39*). If SDS-stable oligomers of Aβ in cell media were principally formed from secreted monomers, then under conditions in which secreted monomeric Aβ (but not intracellular Aβ) is rapidly degraded, oligomer levels should fall. The fact that they do not (*35, 36, 39*) provides further evidence that these oligomeric species arise intracellularly. In addition, the visualization of Aβ dimers very early in the course of our pulse−chase experiments suggests they can arrive in the medium already formed. In view of the apparent stability of the extracellular oligomers we describe, it is particularly intriguing that Roher et al. have shown that monomeric Aβ extracted directly from human senile plaques can readily polymerize to form fibrils upon in vitro incubation, whereas stable dimers and trimers of Aβ isolated from such senile plaques under identical conditions do not go on to form fibrils but are nonetheless neurotoxic (*17*). It should be noted that while extracellular Aβ monomer present in our culture system did not oligomerize upon incubation, the Aβ monomer present in the brain might undergo extracellular

oligomerization under the influence of pro-aggregating factors not present in our cultures.

Intraneuronal accumulation of Aβ-immunoreactive species has been demonstrated both in culture (*21*) and in vivo (*13*, *14*), but because the techniques employed (ELISA and immunohistochemistry, respectively) cannot discriminate among different assembly forms of Aβ, it has heretofore been impossible to conclude whether the species detected included oligomers. The Aβ oligomers we now demonstrate were recovered from cells by lysis in 1% NP-40 buffer (Figure 4) or 1% Triton X-100 (not shown), whereas the intracellular Aβ that was recovered by Skovronsky et al. from NT2N cells (*21*) included both species that were detergent-soluble and some less soluble species that required extraction into formic acid. Although these authors observed no time-dependent accumulation of detergent-soluble Aβ in the NT2N cells (*21*), this does not preclude the accumulation of Aβ oligomers in the detergent-soluble fraction, as it has been shown that the Aβ ELISA employed in their study is incapable of detecting SDS-stable oligomers of Aβ (*40*). Interestingly, the latter study (*40*) describes a 7−8 kDa Aβ-immunoreactive protein in lysates of a neuronal (SY5Y) cells that is Triton-insoluble and resembles the dimeric species described here. We have evidence that the oligomers we describe here are not dissociated by 70% formic acid (D.M.W. and D.J.S., unpublished data), suggesting that these species would be unlikely to be detected by ELISA even after formic acid treatment such as used by Skovronsky et al. (*21*).

Evidence for the potential biological significance of the SDS-stable Aβ oligomers we describe comes both from their augmentation by AD-causing presenilin or APP mutations (*20*) and from their presence in some human CSF samples (Figure 1C). The latter finding is consistent with a previous report that small amounts of stable, low-*n* oligomers of Aβ can be found in human CSF (*34*). Using a combination of affinity chromatography and laser atomic desorption mass spectrometry to examine a 3 L pool of human CSFs, Vigo-Pelfrey et al. detected dimers of Aβ1−40 and apparent trimers of Aβ1−35 and Aβ6−42 (*34*). Whereas Aβ levels in human CSF have been extensively investigated using ELISA [for an overview, see (*41*)], almost no effort has been made to search for different assembly forms of Aβ in CSF, some of which might be poorly or not detectable by ELISA (*40*), and to investigate their relationship to AD. Although in the current study we observed that certain human CSF samples contained SDS-stable oligomers of Aβ while others did not, further analyses are required to determine if oligomer detection correlates with age or presence of AD and whether even more sensitive methods would detect oligomers more commonly. In some of our CSF samples, the background in the gel region of interest (6−14 kDa) was sufficiently high to necessitate the use of preimmune serum to confirm the authenticity of the Aβ immunoreactive bands. An ideal comparison of CSF from AD and non-AD patients will thus require sufficient volumes to allow for immunoprecipitation of each sample with one or more anti-Aβ antibodies versus preimmune serum.

In a recent study of 14 patients with clinically diagnosed AD and 19 patients with other neurological disorders, a strong correlation between the presence of AD and the appearance of large putative Aβ aggregates in the CSF was reported (*42*). In that study, CSF was incubated with nanomolar concentrations of fluorescently labeled synthetic Aβ1−42, and interactions of this probe with endogenous Aβ species were detected by fluorescence correlation spectroscopy. Interactions of the fluorescent probe with large structures in the CSF were greatly diminished by prior immunodepletion of the fluid with an anti-Aβ antibody, suggesting that the structures with which the probe interacted were either aggregates of Aβ or macromolecules which could coimmunoprecipitate with Aβ. Our inability to visualize the kinds of large structures reported by Pitschke et al. (*42*) in our CSF samples may mean that these structures do not enter SDS−PAGE, or they comigrate with higher molecular weight APP isoforms which are recognized by the Aβ antibodies we use, or they comigrate with nonspecific proteins (e.g., immunoglobulins and their fragments) that are apparent in the higher molecular weight regions of our Western blots.

Because young transgenic mice can lack amyloid plaques but exhibit intraneuronal accumulation of Aβ42 and signs of neurodegeneration (*12*, *13*), it will be important to seek a correlation between levels of SDS-stable Aβ oligomers such as we describe and neurodegeneration in mouse and human brain tissue and in primary neuronal cultures of increasing ages. From a therapeutic standpoint, there is growing evidence that, in addition to the extensively characterized fibril-related neurodegeneration seen in culture models and in vivo, prefibrillar (oligomeric and polymeric) species of Aβ may well play a role in AD pathogenesis (*9*−*13*). Before rational anti-aggregation therapies can be developed, the pathogenic roles of nonamyloid (i.e., prefibrillar) forms of Aβ both intracellularly and extracellularly should be clarified. The possibility that certain anti-aggregation agents could elevate the levels of potentially toxic Aβ intermediates must be carefully considered. In view of the new findings we report here, our cell culture model system should be advantageous for further examining the mechanism of formation of intracellular Aβ oligomers and screening for their therapeutic inhibition.

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