Articles

Oligomerization of Endogenous and Synthetic Amyloid β-Protein at Nanomolar Levels in Cell Culture and Stabilization of Monomer by Congo Red[†]

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ABSTRACT: Amyloid β-proteins (Aβ) are proteolytic fragments of the β-amyloid precursor protein (βAPP) that are secreted by mammalian cells throughout life but also accumulate progressively as insoluble cerebral aggregates in Alzheimer's disease (AD). Because mounting evidence indicates that Aβ aggregation and deposition are early, critical features of AD leading to neurotoxicity, many studies of Aβ aggregation have been conducted using synthetic peptides under generally nonphysiological conditions and concentrations. We recently described the oligomerization of Aβ peptides secreted by βAPP-expressing cells at low nanomolar (20−30 ng/mL) levels into sodium dodecyl sulfate- (SDS-) stable oligomers of 6−16 kDa. Here, we extensively characterize this in vitro system and show that the amyloid binding dye, Congo red, acts to markedly decrease oligomer/monomer ratios by stabilizing the 4 kDa Aβ monomers ($ID_{50} \cong 3.4$ μM). Addition of radioiodinated synthetic Aβ_{1-40} to the cultures or to their conditioned media at physiological concentrations (0.25−2.5 nM) reveals that it undergoes progressive aggregation into SDS-stable oligomers of 6−25 kDa during brief (∼4 h) incubation at 37 °C, and this is inhibitable by Congo red. The level of Aβ oligomers can be quantitated in the Chinese hamster ovary (CHO) conditioned medium by size-exclusion chromatography as well as by SDS−polyacrylamide gel electrophoresis (PAGE), and comparison of these two methods suggests that aggregation of Aβ into higher molecular weight polymers that are not detectable by SDS−PAGE occurs in the cultures. We conclude that both endogenous and synthetic Aβ can assemble into stable oligomers at physiological concentrations in cell culture, providing a manipulable system for studying the mechanism of early Aβ aggregation and identifying inhibitors thereof under biologically relevant conditions.

Converging evidence from numerous genetic, neuropathological, biochemical, and animal modeling studies points to a central role for the amyloid β-protein (Aβ)[1] in the pathogenesis of Alzheimer's disease (AD). All four genes implicated to date in familial forms of AD have been shown either to increase the cellular production of the highly amyloidogenic Aβ_{1-42} peptide [in the case of the β-amyloid precursor protein (βAPP), presenilin 1, and presenilin 2 genes (*1*−*8*)] or to increase the number and density of cerebral deposits of Aβ [in the case of the apolipoprotein E4 allele (*9*−*13*)]. Aβ constitutes a group of proteolytic fragments with heterogeneous N- and C-termini that are cleaved from βAPP and secreted by both neural and nonneural cells throughout life (*14*−*17*). Whereas soluble Aβ peptides are normal metabolic products, excessive accumulation of the peptides in the extracellular space of the brain can apparently lead to their gradual aggregation into insoluble nonfilamentous deposits (diffuse plaques) (*18*, *19*) and filamentous deposits (senile plaques). The latter are intimately associated with degenerating axons and dendrites, activated microglia, and reactive astrocytes. These in vivo events can be mimicked in part in vitro, in that synthetic Aβ peptides in the aggregated but not the monomeric state injure cultured neurons and activate microglia and astrocytes (e.g., refs *20*−*22*). Moreover, transgenic mice overexpressing AD-linked βAPP mutations show an age-dependent development of fibrillar Aβ deposits associated with degenerating neurites, activated microglia, and reactive astrocytes (*23*−*25*).

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[1] Abbreviations: Aβ, amyloid β-protein; βAPP, β-amyloid precursor protein; AD, Alzheimer's disease; IAβ_{40}, radioiodinated Aβ_{1-40}; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CR, Congo red; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; SEC, size-exclusion chromatography.

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Because Aβ aggregation appears to be a critical step for conferring cytotoxicity, many studies of this process have been conducted using synthetic peptides of a single defined length, generally under nonbiological conditions and concentrations. To determine whether Aβ aggregation can be observed in a more physiological system, we searched for and found evidence of the oligomerization of the endogenous Aβ peptides that are constitutively released into the medium of cultured cells at nanomolar concentrations (*26*). In our initial study, soluble, SDS-stable peptides of 6−16 kDa were immunoprecipitated from the conditioned media of ^{35}S-methionine-labeled Chinese hamster ovary (CHO) cells expressing βAPP and confirmed as Aβ oligomers by both selective immunoreaction and protein sequencing (*26*). Furthermore, we showed that the oligomer/monomer ratio could be decreased by adding the amyloid-binding dye, Congo red (CR), during labeling of the cells.

We have now extended these findings and developed them into a manipulable in vitro model system useful for studying the initial steps in Aβ aggregation and as a screen to identify and characterize inhibitors thereof. Addition of radioiodinated Aβ_{1-40} peptide at physiological (0.25−2.5 nM) concentrations to the cultures reveals that it undergoes an oligomerization process similar but not identical to that of the endogenous peptide, and this is inhibitable by CR. Systematic examination of how CR markedly decreases the oligomer/monomer ratio in cultures shows that the drug acts principally by stabilizing the monomer from subsequent oligomerization rather than by substantially altering Aβ production or clearance. We conclude that the progressive assembly of Aβ into oligomers and probably higher polymers can occur in cell culture, supporting the utility of this system for studies of the mechanism and pharmacological inhibition of Aβ aggregation under biologically relevant conditions.

MATERIALS AND METHODS

βAPP-Expressing Cell Lines. Chinese hamster ovary (CHO) cells stably transfected with a βAPP$_{751}$ cDNA containing the Val717Phe mutation (residue 46 by Aβ numbering; designated CHO$_{V717F}$ cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone), as previously described (*26*).

Antibodies. R1282, a rabbit antiserum raised against synthetic human Aβ_{1-40} (*14*), was used at a dilution of 1:75 for immunoprecipitation of conditioned medium. Monoclonal antibody 21F12 raised against synthetic Aβ_{31-42} is specific for Aβ species ending at residue 42 (*27*). 21F12 was provided by P. Seubert and D. Schenk (Athena Neurosciences) and was used at 7.5 μg/mL for immunoprecipitation.

Immunoprecipitation and Gel Fluorography. Nearly confluent (95−100%) 10 cm dishes of CHO$_{V717F}$ cells were labeled with [^{35}S]methionine and immunoprecipitated as previously described (*26*), except as noted. For all experiments, the cells were labeled in serum-free DMEM. CR (Aldrich, Milwaukee, WI) was prepared as a stock solution of 10 mM (or 100 mM for some dose-finding studies) in either dimethyl sulfoxide (DMSO) or water (results in each solvent were indistinguishable) and added to culture medium at a 1:1000 dilution during metabolic labeling. For pulse−chase experiments, cells were pulsed with 300 μCi of [^{35}S]-methionine in methionine-free medium for 2 h and then washed and chased in serum-free medium with or without CR. For pulse−chase experiments examining initial oligomer production, cells were treated as above but pulsed with 1 mCi of [^{35}S]methionine for just 10 min prior to chasing. For Aβ cellular uptake experiments, the conditioned medium of metabolically labeled cells was centrifuged at 2100*g* for 30 min and the supernatant was incubated with unlabeled CHO cells for ∼18 h. Protease inhibitors added to the conditioned medium for some experiments were 1 μM E-64, 10 μM leupeptin, 1 μM pepstatin A, 2 μg/mL aprotinin, and 0.1 mM Pefabloc. For Aβ degradation experiments, 2 h labeled medium (as a source of radiolabeled endogenous Aβ) and overnight unlabeled medium (as a source of proteases) were each spun as above, and the supernatants were combined and incubated at 37 °C with gentle agitation for the indicated times.

[^{125}I]Aβ_{1-40} Experiments. Human Aβ_{1-40}-OH was synthesized, characterized, purified and radioiodinated at residue tyrosine 10, as previously reported (*28*). No differences in aggregation properties were seen among the three batches of iodinated peptide (IAβ_{40}) used, as judged by SDS−PAGE and size-exclusion chromatography (SEC), except as specifically noted in Results. Each peptide batch was used for less than 30 days after its iodination, as anomalous behavior was occasionally observed thereafter. The specific activity of IAβ_{40} was ∼2000 Ci/mmol (10^9 cpm/μg), and the stock was ∼50 nM in 35% acetonitrile/0.1% triflouroacetic acid/0.1% β-mercaptoethanol. Tissue culture dishes and tubes were precoated with DMEM/10% FBS and rinsed with DMEM to minimize nonspecific sticking of IAβ_{40}. CHO cells (untransfected or CHO$_{V717F}$) were grown to ∼90−100% confluence in 3.5 cm dishes, rinsed with serum-free DMEM, and cultured at 37 °C with IAβ_{40} diluted to 1:200 (∼0.25 nM) from stock in the desired medium (serum-free DMEM with or without 10 μM CR). Alternatively, serum-free conditioned medium from CHO cells (untransfected or CHO$_{V717F}$) was collected after ∼15−18 h conditioning and spun at 6000*g* for 5 min, and the supernatant was incubated with the IAβ_{40} in microfuge tubes at 1:200 in the presence or absence of 10 μM CR. For SDS−PAGE analysis, aliquots of conditioned medium were removed from a dish or tube at indicated time intervals, mixed with sample buffer, stored at 4 °C, and then boiled for 5 min or incubated at 80 °C for 10 min, spun briefly, and electrophoresed on 10−20% polyacrylamide Tris−tricine gels (Novex) and subjected to autoradiography. Samples were also subjected to scintillation counting to ascertain total counts per minute. For size-exclusion chromatography, aliquots were removed at time 0 and 4.5 h from the same dish (experiments with cells present) or tube (experiments without cells present), spun at 6000*g* for 5 min, and immediately fractionated (see below). In experiments in which the fractionated samples were also evaluated by SDS−PAGE, a 10-fold higher concentration of IAβ_{40} (∼2.5 nM) was used, and the fractionated samples were immediately electrophoresed on 16% polyacrylamide Tris−tricine gels.

Size-Exclusion Chromatography. Size-exclusion columns with bed volumes of ∼21 mL were prepared using Sephadex G-50 superfine medium (Pharmacia) in borosilicate Econo-columns (30 × 1 cm, Bio-Rad). Columns were eluted at a

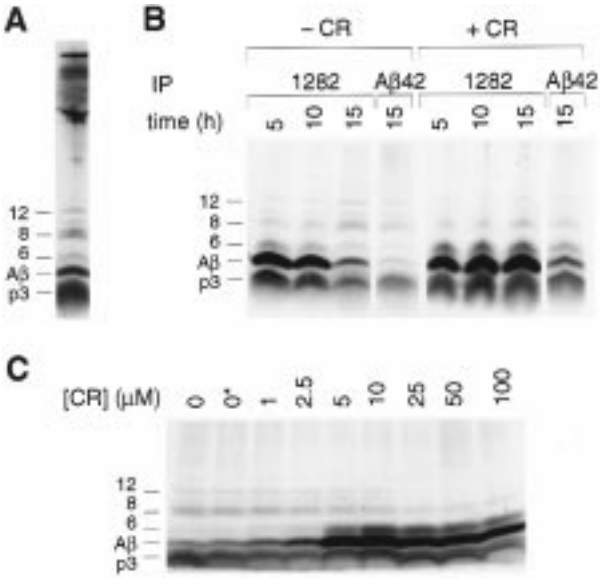

FIGURE 1: Effect of Congo red (CR) on Aβ monomer and oligomers in metabolically labeled CHO cells. Immunoprecipitates of Aβ species from conditioned media of [^{35}S]Met-labeled CHO_{V717F} cells were electrophoresed on 16% polyacrylamide Tris−tricine gels. Positions of various Aβ species are indicated on the left. (A) Antiserum 1282 immunoprecipitation (IP) after overnight labeling shows characteristic SDS-stable Aβ species migrating from 3 to 12 kDa. (B) 1282 IP after 5, 10, or 15 h of labeling reveals a marked decrease of Aβ monomer and a modest increase in Aβ oligomers over time in the absence (−) but not in the presence (+) of 10 μM CR. IP of $A\beta_{42}$ by antibody 21F12 reveals the presence of this peptide in the oligomeric species. (C) 1282 IP of medium after overnight labeling in the presence of 0−100 μM CR demonstrates maximal increase of Aβ monomer at 10 μM. 0*, no CR present during conditioning but 50 μM CR added just prior to IP.

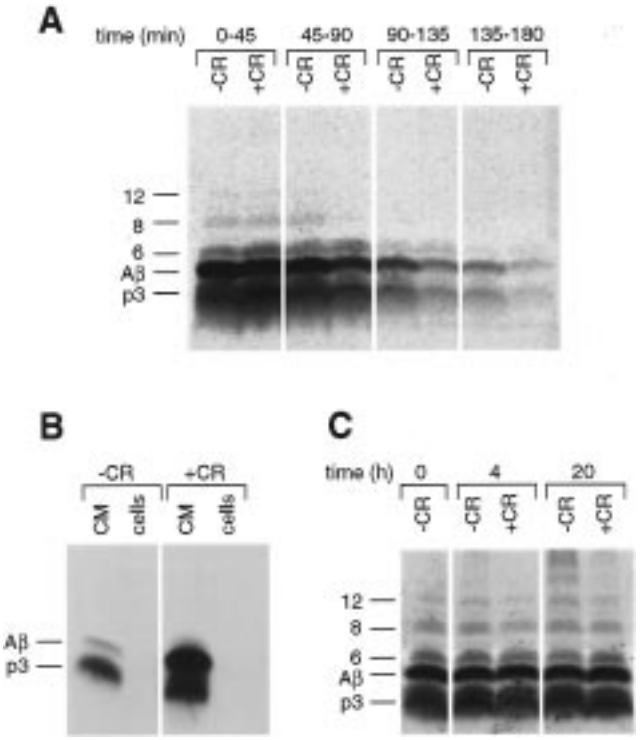

FIGURE 2: Effect of Congo red on Aβ production, uptake, and stability: 1282 immunoprecipitations of [^{35}S]Met-labeled CHO_{V717F} conditioned media or cell lysates. (A) Conditioned media from cells pulsed for 2 h and chased in 45 min intervals in the presence (+) or absence (−) of 5 μM CR show that CR modestly decreases Aβ levels. (B) IP of conditioned medium (CM) or cell lysate (cells) from unlabeled CHO cells after overnight incubation in 2 h radiolabeled conditioned medium. Absence of signal in the cell lanes indicates that no detectable uptake of labeled Aβ occurred in the presence or absence of 10 μM CR. (C) Labeled medium (for 2 h) from CHO_{V717F} cells was mixed with unlabeled overnight-conditioned CHO medium (as a source of proteases) and incubated at 37 °C for 0, 4, or 20 h in the presence (+) or absence (−) of 10 μM CR.

linear flow rate of ∼12.9 cm/h with 0.1 M Tris-HCl, pH 7.4, and 0.02% sodium azide, using a peristaltic pump (LKB Microperpex S). Absorbance was monitored at 254 nm using a Pharmacia UV-1 single path monitor, fractions (250 μL) were collected, and the presence of [^{125}I]$A\beta_{1-40}$ was determined by scintillation counting. Freshly prepared columns were used for each experiment to prevent possible interference due to accumulation of peptide or CR.

RESULTS

Immunoprecipitation of the serum-free conditioned medium of ^{35}S-methionine-labeled CHO_{V717F} cells with Aβ antibodies revealed the presence of SDS-stable oligomeric Aβ species migrating at ∼12, 10, 8, and 6 kDa in addition to the 4 kDa (Aβ) and 3 kDa (p3) monomers (Figure 1A) (*26*). p3 is a peptide comprising residues 17−40 (or 42) of Aβ that results from constitutive proteolysis of βAPP by α- and then γ-secretases, whereas Aβ (1−40 or 1−42) results from constitutive proteolysis of βAPP by β- and then γ-secretases (reviewed in ref *29*). To examine the relationship of monomers to oligomers over time, conditioned media of CHO_{V717F} cells were collected after serum-free metabolic labeling for 5, 10, or 15 h, immunoprecipitated with R1282 (a polyclonal antibody to synthetic human $A\beta_{1-40}$), and electrophoresed on Tris−tricine gels. Beginning around 10 h of labeling, we observed a time-dependent decrease in the 3, 4, and 6 kDa bands in the media accompanied by a small increase of the 8−12 kDa oligomeric bands (Figure 1B). [The exact nature of the 6 kDa species is unknown; radiosequencing previously showed that it contains Aβ beginning at Asp_1 (*26*), so that it may represent an unusually folded form of Aβ monomer (or possibly Aβ dimer) that migrates anomalously in SDS−PAGE.] The presence of Congo red (10 μM) during the labeling completely prevented these changes (Figure 1B). When the media were precipitated with monoclonal antibodies highly specific for Aβ peptides ending either at residue 42 ($A\beta_{42}$) (Figure 1B) or residue 40 ($A\beta_{40}$) (not shown), essentially identical results were obtained. This finding shows that secreted $A\beta_{42}$ is present in these low-molecular weight oligomers and that it behaves similarly to the more abundantly secreted $A\beta_{40}$ peptide. To establish the earliest time point at which endogenous Aβ oligomers are detectable in the media, cells were pulse-labeled for 2 h and then chased for increasing intervals between 30 and 180 min in serum-free medium. The 8−12 kDa oligomers were detected after as little as 45 min of chase (Figure 2A). When the cells were pulse-labeled for just 10 min and the chase medium was exchanged every 15 min, we first observed the 3, 4, and 6 kDa bands at ∼30−45 min and the 8−12 kDa Aβ oligomers at ∼60 min of chase (not shown). Taken together, these experiments suggest that freshly secreted Aβ peptides begin to oligomerize within 1 h in CHO cultures and that CR can strongly stabilize the monomers.

We noted that the progressive decrease in monomer observed after prolonged (>8 h) labeling was always quantitatively greater than the simultaneous increase in oligomers in our experiments (Figure 1B). One trivial explanation for this discrepancy could be that our antibodies immunoprecipitate oligomers less efficiently than monomers. We therefore determined the concentration of antiserum R1282 required to achieve maximal precipitation of the oligomers. As our standard R1282 dilution of 1:300 was decreased to 1:75 or less, the amount of Aβ monomer precipitated did not change significantly but that of the oligomers increased (not shown). Using R1282 at 1:75, we then quantitated by phosphorimaging the effects of CR on Aβ monomer and oligomer levels in overnight metabolic labeling experiments. Compared to the situation in plain medium, the inclusion of CR during labeling increased the levels of the 4 kDa Aβ monomer ~9-fold ($p < 0.005$, $n = 9$) and of the 6 kDa band ~10-fold ($p < 0.002$, $n = 9$), while decreasing the oligomers slightly and insignificantly ($p = 0.1$, $n = 9$). As a result, CR decreased the percentage of the total precipitable Aβ gel signal represented by the oligomers from 32% to 3.5% ($p < 0.0001$, $n = 9$). We next examined increasing doses of CR between 1 and 100 μM for their effects on Aβ monomer levels (Figure 1C). As quantitated by phosphorimaging, the degree of stabilization of Aβ monomers was dose-dependent between 1 and 10 μM, with the ED_{50} in this system being 3.4 μM, corresponding to a molar ratio of Aβ monomer:CR of ~1:300. No further monomer increase occurred at CR doses above 10 μM.

In view of the discrepancy between the large CR-mediated increase in Aβ monomer and the small decrease in the 8−12 kDa oligomers visualized by SDS−PAGE, we searched systematically for possible causes of the sharp rise in monomer other than inhibition of its oligomerization. First, we examined the effect of CR on Aβ production by the cells. Using a pulse−chase paradigm, CR was found to modestly decrease rather than increase Aβ production (Figure 2A). This effect was associated with a corresponding modest increase in the amounts of the ~12 and 14 kDa C-terminal fragments of βAPP in lysates in the presence of CR (data not shown), suggesting that CR may modestly inhibit the γ-secretase cleavage of βAPP. CR produced no change in full-length βAPP in lysates or $βAPP_s$ in media. Preincubation of the cells in CR for up to ~15 h prior to pulse labeling still resulted in a modest decrease in Aβ production (data not shown), ruling out a delayed increase in Aβ production as the basis for the sharp rise in Aβ monomer levels that CR consistently produced in metabolic labeling studies. Next, to examine whether CR alters any putative cellular reuptake of Aβ, we incubated 2 h labeled medium from the CHO_{V717F} cells with nontransfected CHO cells in the presence or absence of CR for ~15 h. Subsequent R1282 immunoprecipitation revealed abundant labeled Aβ in the medium (Figure 2B, lanes 1 and 3) but none in cell lysates, whether CR was present or not (lanes 2 and 4). To confirm this result, we also incubated the cultures with $[^{125}I]Aβ_{1-40}$ (0.25 nM) with or without CR and noted virtually no uptake of the tracer (not shown). The fact that CR was again associated with substantially increased levels of labeled Aβ in the medium in these experiments (Figure 2B, lanes 1 vs 3) in the absence of any possible new production of labeled Aβ confirmed that a CR-mediated increase in Aβ production cannot be the basis for the elevation. The presence of serine, cysteine, and aspartate protease inhibitors in the medium (see Materials and Methods) did not alter this result, arguing against the possibility that CR inhibits a cell-surface protease capable of quantitatively degrading Aβ.

Another possible mechanism for the CR effect is that it inhibits the degradation of Aβ in the medium by a secreted protease. To address this possibility, 2 h labeled medium from CHO_{V717F} cells (as a source of labeled Aβ) was mixed with 16 h conditioned medium of untransfected CHO cells (as a source of secreted proteases), incubated at 37 °C for prolonged periods in the presence or absence of CR, and the immunoprecipitated samples were quantitated by phosphorimaging. After a 20 h incubation, we observed a ~40% ($n = 4$) decrease in Aβ monomer levels in the absence of CR, and this was not significantly altered by the presence of CR (Figure 2C). This is in contrast to the ~90% decrease of Aβ monomer seen in the absence of CR during our standard overnight labeling paradigm. Thus, any inhibition of a secreted Aβ-degrading protease by CR is by itself insufficient to fully explain the large rise in Aβ monomer levels we routinely observe when CR is present during metabolic labeling experiments in our system.

In view of the above results and the fact that CR has been shown to inhibit the aggregation and in vitro neurotoxicity of synthetic Aβ (*20*, *30*) and the aggregation and infectivity of the prion protein in cell culture (*31*, *32*), the most plausible explanation for the major portion of the CR-induced rise in Aβ monomer levels in our cultures is that the compound inhibits Aβ aggregation. To confirm this directly and also address whether additional Aβ oligomers and polymers form in the medium that are not detectable by immunoprecipitation/SDS−PAGE, we performed experiments using [^{125}I]-labeled $Aβ_{1-40}$ ($IAβ_{40}$) as a tracer in the cultures and analyzed the results by SDS−PAGE without immunoprecipitation as well as by size-exclusion chromatography (SEC). Physiologic amounts of $IAβ_{40}$ (~0.25 nM) were added to the cultures, and aliquots of conditioned medium were collected from the same dish at increasing time intervals between 0 and 8 h, separated on Tris−tricine gels, and examined by autoradiography. Incubation of $IAβ_{40}$ in unconditioned DMEM alone at 37 °C revealed only the 4 kDa monomeric band that remained stable over time (Figure 3A). In contrast, incubation of $IAβ_{40}$ in the CHO cultures led to a graded decrease in the 4 kDa monomer beginning as early as 30 min, accompanied by the appearance and gradual increase of SDS-stable oligomers migrating between ~6 and ~ 25 kDa (Figure 3C). This concurrent loss of monomer and gain of oligomers between 0 and 4 h was quantitated by phosphorimaging (see, e.g., Figure 3B, top panel). The Aβ monomer level decreased from a mean of 93% of the total initial Aβ gel signal to 30% at 4 h ($p = 0.009$, $n = 3$), while Aβ oligomer levels increased from 7% to 16% ($p = 0.09$) of the initial gel signal. Figure 3B (top panel) also shows that while the *total* Aβ signal in the gels decreased over time, the oligomer/monomer ratio rose steadily from 9% at 0 h to 48% at 8 h (see also below).

To determine whether the aggregation of $IAβ_{40}$ in the CHO cultures was due to the presence of the cells themselves or to substances released by the cells, $IAβ_{40}$ was incubated solely in overnight-conditioned medium and assayed as above. In this cell-free incubation, $IAβ_{40}$ again existed

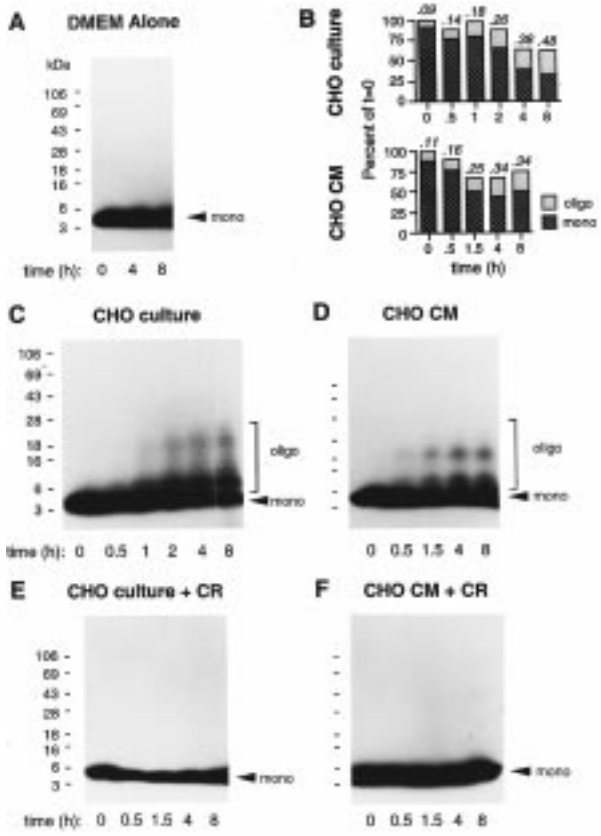

FIGURE 3: Incubation of 250 pM [^{125}I]Aβ_{1-40} with CHO$_{V717F}$ cell cultures or their conditioned media at 37 °C for the indicated times (in hours) results in the decrease of monomer and appearance of SDS-stable oligomers, as revealed by direct electrophoresis on 10−20% polyacrylamide Tris−tricine gels. MW standards are indicated on the left and the positions of IAβ_{40} monomer and oligomers on the right. (A) Incubation in plain DMEM does not alter the IAβ_{40} monomer. (B−D) Incubation of IAβ_{40} with CHO cultures (panel C) or in overnight CHO conditioned medium (CM) alone (panel D) results in loss of monomer and concurrent increase in oligomers in the medium. Panel B shows the quantitative phosphorimaging of gels in panels C (top) and D (bottom); bars represent signal detected in the monomer (mono) or oligomer (oligo) position expressed as a percent of the total signal at time 0. Numbers in italics at tops of bars are the ratios of oligomer/(oligomer + monomer) at each time point. (E, F) Addition of 10 μM CR to whole CHO cultures (panel E) or their CM alone (panel F) fully inhibits the loss of monomer and increase of oligomers.

initially as a 4 kDa monomer that diminished over time (starting at 30 min), while SDS-stable oligomers migrating from ~6 to 25 kDa appeared and increased in amount (Figure 3D). Thus, factors released by the CHO cells were sufficient to allow IAβ_{40} aggregation. Quantitation by phosphorimaging confirmed that incubation of IAβ_{40} in conditioned medium alone caused a loss of Aβ monomer from 90% of the initial gel signal to 50% at 4 h ($p < 0.001$, $n = 5$), while the Aβ oligomers increased from 10% to 18% ($p = 0.01$) (see, e.g., Figure 3B, bottom panel). The presence of 10 μM CR during the incubation of IAβ_{40}, either in whole cultures or in their conditioned medium alone, consistently prevented the decline in the 4 kDa monomer and the appearance of the oligomers (Figure 3E,F). The fact that the decrease in monomer occurred with IAβ_{40} in conditioned medium in the absence of cells further discounted the theoretical possibility that CR is principally inhibiting an Aβ-degrading cell-associated protease.

When we incubated the IAβ_{40} with nontransfected CHO cells or their conditioned media, a similar but slightly less robust oligomerization of the tracer was seen, suggesting that factor(s) in the medium other than (or in addition to) secreted Aβ help mediate the in vitro oligomerization process. To rule out any residual effect of the small amounts of Aβ secreted by these nontransfected cells, their overnight-conditioned medium was incubated with either R1282 or preimmune serum, the resulting immune complexes were precipitated with protein A−sepharose, and the remaining supernatant was then incubated with IAβ_{40} as before. No difference in the IAβ_{40} monomer and oligomer patterns occurred, supporting the notion that a secreted cellular factor other than Aβ itself is principally involved in the aggregation. In the experiments utilizing IAβ_{40} as in those examining endogenous Aβ, we consistently observed a major decrease in Aβ monomer in the gels that occurred concurrently with the appearance of minor amounts of SDS-stable oligomers. These data support the hypothesis that additional aggregates of endogenous Aβ and of IAβ_{40} are present in the medium that are not detectable by SDS−PAGE.

To address this hypothesis, we attempted to separate the Aβ aggregates and detect them without using SDS−PAGE, namely, by employing IAβ_{40} in size-exclusion chromatography (SEC) of medium. First, IAβ_{40} added to plain DMEM was immediately fractionated on a G50 SEC column and the fractions were assayed by counting radioactivity (Figure 4A). Two distinct peaks were consistently detected: the first, called peak 1 (eluting at a molecular mass of ~4 kDa), was found to be reproducible among all peptide batches and was tentatively interpreted to be the IAβ_{40} monomer peak, whereas the second, called peak 3, was very small but differed in size among batches of IAβ_{40} and was interpreted to contain species of IAβ_{40} that arose from degradation and/or aggregation of the peptide while in storage. In support of this interpretation, early experiments showed that peak 3 was larger when IAβ_{40} was used after 30 days of storage at −20 °C, coinciding with gel analyses of the stock peptide that showed that SDS-stable aggregation had indeed occurred. Subsequently, IAβ_{40} batches were always used prior to 30 days of storage and monitored by SDS−PAGE for aggregation.

Incubation of the IAβ_{40} in plain DMEM for 0 or 4.5 h at 37 °C resulted in very little or no change in the SEC pattern (Figure 4A). In contrast, incubation of IAβ_{40} in the CHO$_{V717F}$ cultures for 4.5 h resulted in a striking decrease in peak 1 and the concurrent appearance of a new peak, designated peak 2 (Figure 4B). This result was reminiscent of the decrease of IAβ_{40} monomer and appearance of oligomers seen previously on SDS gels (Figure 3C). To obtain evidence that peak 2 contained IAβ_{40} aggregates, we incubated IAβ_{40} in the cultures for 4.5 h in the presence of 10 μM CR. This resulted in an identical profile to that observed by incubating in plain DMEM (i.e., peak 1 but not peak 2), demonstrating that CR fully inhibited the change in chromatographic behavior of IAβ_{40} (Figure 4C). To test whether soluble factors released by the cells could cause this change, IAβ_{40} was incubated in overnight-conditioned medium alone for 0 or 4.5 h and assayed as above. Following the 4.5 h incubation, a reproducible modest reduction in peak 1 was accompanied by the appearance of peak 2 (Figure 4D).

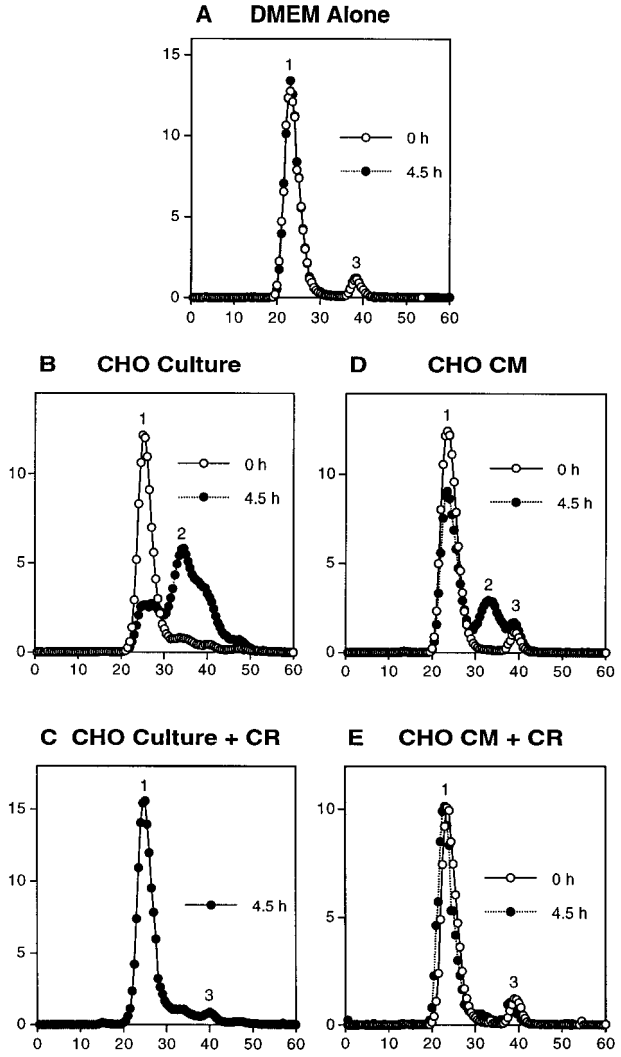

FIGURE 4: Size-exclusion chromatography (SEC) of 250 pM [^{125}I]Aβ1−40 incubated for 0 or 4.5 h with CHO_{V717F} cell cultures or their conditioned media (CM). Radioactivity of fractions separated on Sephadex G50 is graphed, with fraction number on the abscissa and counts per minute × 10^3 on the ordinate. Samples were incubated as in Figure 3, spun at 16000*g* for 5 min, and immediately loaded onto a SEC column. (A) IAβ_{40} incubated in DMEM alone resulted in two peaks that did not change with incubation time. Molecular mass standards eluted at the following fraction numbers: 17 kDa, fraction 16; 12.4 kDa, fraction 18; 6.5 kDa, fraction 23; and 1.3 kDa, fraction 31. (B) Incubation of IAβ_{40} in the CHO cultures results in the appearance of a new peak (2) and a concomitant decrease in the first peak (1). (C) Addition of 10 μM CR blocks this change. (D) Incubation in CM alone also leads to a decrease in peak 1 and the appearance of peak 2. (E) Addition of 10 μM CR blocks this change.

Addition of 10 μM CR during this incubation again blocked the conversion (Figure 4E).

To verify the assigned identities of the three peaks, 2.5 nM IAβ_{40} was added to conditioned media, the samples were incubated at 37 °C and separated by G50 SEC, and aliquots of the resultant fractions were immediately visualized on Tris−tricine gels as well as counted for radioactivity. At time 0, peak 1 was found to contain an abundant 4 kDa band, while peak 3 contained only a faint 4 kDa signal (Figure 5). After incubation at 37 °C for 4.5 h, peak 1 still contained a 4 kDa band, but the new peak 2 contained SDS-stable oligomers of IAβ_{40} migrating predominantly at 8 kDa but also at ~25, 16, and 6 kDa, plus some monomeric 4 kDa

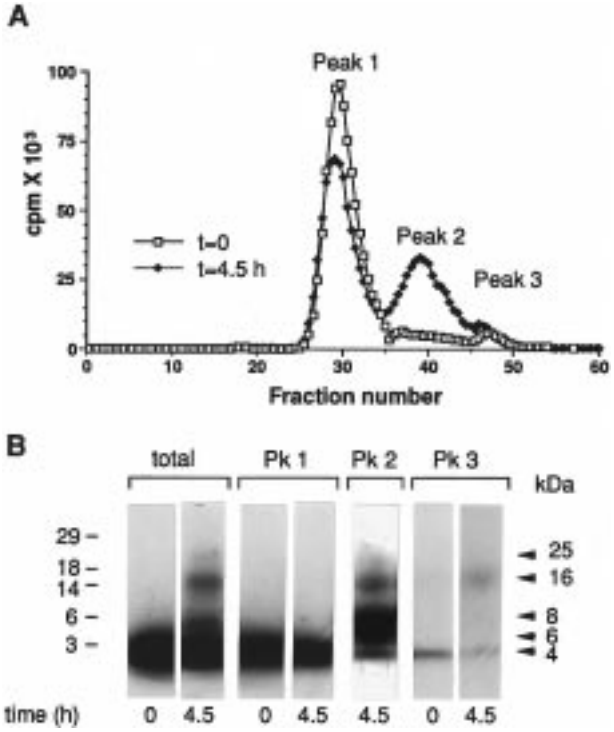

FIGURE 5: SDS-PAGE of size-exclusion fractions of [^{125}I]Aβ_{1-40} incubated in overnight-conditioned CHO_{V717F} medium confirms the occurrence of oligomerization. IAβ_{40} (2.5 nM) was added to conditioned medium for 0 or 4.5 h and then fractionated as in Figure 4, counted, and immediately electrophoresed on 16% polyacrylamide Tris−tricine gels. (A) Graph of scintillation counts reveals characteristic decrease in peak 1 and appearance of peak 2 during 4.5 h. (B) SDS−PAGE of the total sample or representative SEC fractions from each chromatographic peak at times 0 and 4.5 h reveals monomeric species from peak 1 and SDS-stable oligomers from peak 2. A 2 day autoradiographic exposure is shown for the total and peak 1 samples and a 4 day exposure is shown for peaks 2 and 3. MW standards are on the left and approximate sizes of Aβ species are on the right.

Aβ. Peak 3 contained very small amounts of predominantly 16 kDa as well as some 8 and 4 kDa IAβ_{40}. These results demonstrate that the larger Aβ species (oligomers) actually elute at higher fraction number off the SEC column than monomeric Aβ, indicating an anomalous behavior of Aβ oligomers in this chromatographic system, as has been reported for some other amphipathic peptides (*33*, *34*). Therefore, the SDS gels confirmed that we had separated and detected stable IAβ_{40} aggregates in the culture system by a method independent of immunoprecipitation, i.e., by SEC. Importantly, SEC, unlike the denaturing SDS−PAGE system, was able to demonstrate a fully quantitative transition of IAβ_{40} from monomer to aggregates during incubation, i.e., the total counts per minute in all SEC peaks at 4.5 h equaled the total counts per minute at 0 h. This conservation of IAβ counts was consistently observed in SEC experiments using whole cultures or CM alone.

DISCUSSION

Understanding Aβ oligomerization is likely to prove critical for the proper design of small molecules that could reproducibly inhibit this pathogenic process. Here, we provide evidence that both endogenous and synthetic Aβ monomers can undergo a gradual transition into oligomers and probably higher molecular weight polymers in cell

culture at physiological (≤1−3 nM) concentrations and under conditions that resemble those of extracellular fluids.

Monomer−Oligomer Relationships for Endogenous and Synthetic Aβ in Culture. In our studies of endogenous Aβ in CHO cultures, SDS-stable low molecular weight oligomers of secreted Aβ were detectable in the conditioned media of CHO_{V717F} cells after as little as 10 min of pulse labeling and 60 min of cold chase. With increasing intervals of continuous metabolic labeling, the amount of oligomers rose gradually, while the monomers (Aβ and p3) steadily declined. After ∼16 h of labeling, the endogenous oligomer bands in the gels represented ∼30% of the total labeled Aβ species immunoprecipitated from the medium and visualized by SDS−PAGE. To examine this in vitro Aβ aggregation process independently of the continuous production of new monomer that occurs in metabolic labeling experiments, we added $IA\beta_{40}$ to the cultures (or to their conditioned media alone) at physiological concentrations (0.25−2.5 nM) and quantitated any changes over time by phosphorimaging of aliquots electrophoresed on SDS−PAGE. As in the case of the endogenous Aβ, we observed a gradual decline in the amount of monomeric peptide between 30 min and 8 h of conditioning, accompanied by the concurrent appearance of SDS-stable ∼6−25 kDa oligomers. Therefore, CHO cultures, and in particular their conditioned media, allow a progressive oligomerization of exogenous Aβ that is not observed in unconditioned medium after otherwise identical incubation. Quantitation revealed that while the $IA\beta_{40}$ monomer declines steadily in amount throughout an 8 h incubation, the oligomers increase during the early phase (∼2 h) of this period and then remain relatively constant or increase only modestly thereafter (Figure 3B). These data suggest the possibility that the oligomers behave like intermediates which reach steady-state levels and achieve equilibrium with a larger polymeric species. The presence of 10 μM Congo red stabilizes both synthetic and endogenous forms of monomer. It should be noted, however, that these two forms of Aβ do not behave identically. For example, the synthetic and endogenous oligomers do not comigrate precisely on gels, and CR fully prevents the appearance of synthetic oligomers, while endogenous oligomers are still seen when cells are metabolically labeled in the presence of CR. It may not be surprising that the rich array of endogenous Aβ species and related proteins (e.g., p3) found in conditioned media result in a somewhat different oligomerization process (including the formation of the highly stable and apparently CR-resistant Aβ oligomers we see by SDS−PAGE) than occurs with synthetic Aβ of a single, defined length. As discussed below, we hypothesize that the endogenous oligomers we have visualized to date by SDS−PAGE in the cell cultures are unlikely to represent all of the Aβ oligomers/polymers that exist in this system. The ability to simultaneously examine endogenous and exogenous Aβ aggregation in the same in vitro system should be highly advantageous for further studies.

For both types of peptides, we consistently observed a major decrease in monomer that occurred concurrently with the appearance of relatively modest amounts of oligomers, as judged by denaturing gel electrophoresis. This lack of conservation of the total Aβ gel signal over time in this analytical system suggested that substantial aggregation of monomer occurs into forms that cannot be visualized by SDS−PAGE; e.g., they are unstable in SDS. We therefore assayed the in vitro aggregation process by an alternative method, size-exclusion chromatography. During a 4.5 h incubation of $IA\beta_{40}$ in the CHO cultures or in their CM alone, we observed a decline of the monomeric peak and the appearance of a new peak (peak 2) that was shown to contain Aβ oligomers. γ Counting of all the media fractions obtained by SEC after 0 vs 4.5 h of incubation showed a complete conservation of counts; i.e., the $IA\beta_{40}$ lost from peak 1 was quantitatively recovered in peak 2. This result is consistent with the failure to detect degradation products of $IA\beta_{40}$ below the 4 kDa monomer band on gels of the 4.5 h fractions; aggregation but not detectable degradation of the tracer occurs under the conditions and relatively brief duration of this incubation. This conclusion is also consistent with our finding that Aβ-degrading protease(s) secreted by the CHO cells only begin to effect proteolysis of endogenous Aβ after a 6 h incubation at 37 °C (*35*). It is important to note that we do detect low molecular weight degradative products after this longer incubation, demonstrating that our gel system is sensitive for these products.

When SEC peaks 1 and 2 from the 4.5 h incubation of conditioned medium were examined by SDS−PAGE, peak 1 contained solely monomeric Aβ (as it did at 0 h), while peak 2 contained SDS-stable oligomers of ∼6−25 kDa. Integration of the percentage of counts per minute in each SEC peak demonstrated that the oligomerized Aβ species present in peak 2 at 4.5 h represented ∼31% of the total Aβ signal. However, phosphorimaging of SDS−PAGE gels of similarly incubated samples of $IA\beta_{40}$ in conditioned media showed that the oligomers (i.e., the ∼6−25 kDa bands) represented only 18% of the total initial signal. Therefore, separation of oligomerized Aβ species by SDS gel electrophoresis consistently underestimates the amount of aggregation that has occurred (see also ref *36*). Indeed, the major decrease in secreted Aβ monomer observed in CHO conditioned media by SDS−PAGE after 8−10 h of metabolic labeling without a corresponding further increase in the oligomeric gel bands (Figure 1B) suggests that additional aggregation of Aβ into higher molecular weight species that cannot be detected by SDS−PAGE has occurred. The observation that CR, a known inhibitor of synthetic Aβ aggregation, is able to block this decrease of Aβ monomer completely supports this interpretation. It will now be important to fractionate conditioned medium by SEC and attempt to detect endogenous Aβ oligomers by sensitive Aβ enzyme-linked immunosorbent assays in the peak 2 region, where synthetic $IA\beta_{40}$ oligomers were found in the current experiments. These experiments are underway.

Congo Red Stabilizes Aβ Monomer in CHO Cultures by Inhibiting Its Aggregation. In this study, we confirmed our previous observation *(26)* that the addition of Congo red (2−10 μM) to Aβ-secreting CHO cultures maintained without serum dramatically increases the amount of monomer in the medium. We then systematically examined five possible mechanisms by which this CR effect could occur. First, CR did not increase cellular Aβ secretion but rather decreased it modestly. Second, CR did not decrease cellular uptake of Aβ from the medium; indeed, no significant uptake was detected either in the absence or in the presence of CR under our experimental conditions. Third, prolonged incubation of endogenous and synthetic peptides showed that inhibition

of a secreted Aβ-degrading protease by CR could not by itself account for the striking rise in Aβ monomer that we routinely observed. Some degradation of Aβ monomer was observed, but this amount was far less than the ~90% loss of monomer we routinely observe in the absence of CR. Fourth, we obtained no evidence of a cell-associated Aβ-degrading protease inhibitable by CR. This left the fifth possible mechanism; namely, that CR was stabilizing the monomer and thus substantially inhibiting the oligomerization of Aβ. By incubating IAβ_{40} in CHO cultures for 4.5 h and assaying by SEC, we found that the peak containing the monomer decreased over time while a new peak containing ~6−25 kDa oligomers appeared and increased in amount. When CR was present from the beginning of the incubation, the monomer peak remained unchanged and the oligomeric peak did not appear, demonstrating directly that CR blocks the polymerization of exogenous Aβ in cell culture. This finding is consistent with studies of synthetic Aβ in solution, in which CR prevents aggregation of the peptide (*20*, *37*) and thereby blocks its neurotoxicity (*20*, *30*, *38*). Moreover, CR has also been shown to prevent the aggregation and the infectivity of the prion protein in vitro (*31*, *32*). Regarding the mechanism of these effects of CR, we speculate that CR binds Aβ monomers and prevents its interaction with other Aβ monomers and/or with as-yet-unknown factors released by the cells that are capable of promoting Aβ polymerization (see below).

Nature of the Aβ Aggregation Process in Culture. In our initial characterization of the endogenous Aβ oligomers, we determined that they contain Aβ peptides by specific immunoprecipitation with numerous Aβ antibodies and by direct N-terminal protein sequencing, and we obtained some evidence of their N-terminal heterogeneity (*26*). In this study, we extend these observations by demonstrating that Aβ_{42} as well as Aβ_{40} peptides are present in the oligomers (Figure 1B). Previous studies have shown the importance of both N-terminal heterogeneity (*39*) and the Aβ_{42} C-terminus in synthetic Aβ aggregation (*40*−*44*). Indeed, synthetic Aβ species ending at or before Aβ_{40} have generally not been found to form SDS-stable oligomers in test tube experiments (*36*, *40*), and the SDS-stable oligomers detected from synthetic Aβ_{1-42} in such experiments appear to be derived from protofibril or fibril disassembly (*36*). Our studies are consistent with these results, because IAβ_{40} only formed SDS-stable oligomers when incubated in the presence of CHO cultures or their conditioned medium, not in plain DMEM, suggesting the presence of an aggregation-promoting factor(s) (see below). Of particular importance is that our aggregation occurred at physiologic concentrations of Aβ (0.25−2.5 nM), far below the critical concentration for aggregation of synthetic Aβ (micromolar range) (*44*, *45*). Other studies using nanomolar amounts of synthetic IAβ_{40} have similarly found the requirement for a template, seed, or other factors to initiate aggregation at these low concentrations (e.g., refs *28*, *37*, and *46*−*49*). To date, we have identified only soluble, low molecular weight oligomers of Aβ in the CHO conditioned medium. Centrifugation of metabolically labeled conditioned media at 100000*g* for 100 min failed to pellet the oligomers (M.B.P. and D.J.S., unpublished data). The identification of soluble, stable Aβ oligomers ending at both Aβ_{40} and Aβ_{42} has also been made in human brain (*50*, *51*) and cerebrospinal fluid (*52*), supporting the in vivo relevance of the species we have identified in CHO cultures.

A central question raised by our findings concerns the nature of the factors in the CHO cultures that allow the aggregation of secreted and exogenous Aβ at physiological concentrations. In this regard, we consistently find that the conditioned medium alone will promote disappearance of monomer and appearance of oligomers without the need for cells to be present, although the rapidity and extent of the conversion is less than occurs in the presence of cells (Figures 3 and 4). This result indicates that the cells release factors into the medium that promote aggregation and that the cell surface is not absolutely required for the process to occur. One obvious secreted factor that could promote aggregation is Aβ itself; a particular species of Aβ (e.g., a proteolytically cleaved or otherwise modified peptide) could be released into the medium and act as a nidus or "seed" for polymerization of monomer (*39*, *41*, *53*, *54*). Two experimental results argue against an absolute dependence on the levels of a secreted Aβ species in our work. First, the conversion of IAβ_{40} monomer to oligomers occurred in a similar fashion, although to a somewhat lesser degree, in untransfected CHO cultures (and their medium) as it did in transfected cultures that express high levels of the V717F mutant βAPP and thus secrete abundant Aβ. Second, depleting the CHO conditioned medium of soluble Aβ by immunoprecipitating with a general Aβ antiserum did not significantly decrease the subsequent aggregation of IAβ_{40}. However, these experiments do not rule out a role for a secreted Aβ species in promoting aggregation in our system, because the immunodepletion experiments might not substantially remove a particular Aβ species (e.g., a proteolytic fragment) that strongly promotes Aβ assembly. Indeed, we have recently found that CHO cells stably expressing both βAPP and mutant presenilin 1 or presenilin 2 proteins show enhanced Aβ oligomer formation in medium like that described here whenever the elevation of the $AB_{42}/A\beta_{total}$ ratio arising from the mutant presenilin is roughly 2-fold greater than that of wild-type presenilin transfectants (*8*). The latter result strongly suggests that Aβ_{42} levels regulate in part the degree of endogenous Aβ oligomerization seen in our cultures.

Beyond Aβ itself, there could be numerous factors that may act to promote aggregation of the secreted peptide in culture. It is known that low concentrations of metals such as zinc and iron can markedly accelerate the aggregation and precipitation of synthetic Aβ peptides (*46*, *49*, *55*). One or more secreted proteins could promote the assembly of monomers. Presumably, this would not necessarily include extracellular matrix proteins adherent to the culture dishes, because conditioned medium alone allowed oligomerization of Aβ. An important next step in our analysis of this system is to fractionate the conditioned medium of the CHO cultures and determine whether a specific protein or small molecule confers proaggregating properties. We will also search further for higher molecular weight aggregates of endogenous Aβ, including macromolecular assemblies [e.g., amyloid fibrils or protofibrils (*36*, *44*)], in the in vitro system. Finally, these and similar cultures should prove useful as secondary screening systems to characterize and refine novel compounds that inhibit the self-assembly of secreted Aβ peptides into potentially neurotoxic aggregates in Alzheimer's disease.

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