Detection of a Novel Intraneuronal Pool of Insoluble Amyloid β Protein that Accumulates with Time in Culture

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Abstract. The amyloid-β peptide (Aβ) is produced at several sites within cultured human NT2N neurons with Aβ1-42 specifically generated in the endoplasmic reticulum/intermediate compartment. Since Aβ is found as insoluble deposits in senile plaques of the AD brain, and the Aβ peptide can polymerize into insoluble fibrils in vitro, we examined the possibility that Aβ1-40, and particularly the more highly amyloidogenic Aβ1-42, accumulate in an insoluble pool within NT2N neurons. Remarkably, we found that formic acid extraction of the NT2N cells solubilized a pool of previously undetectable Aβ that accounted for over half of the total intracellular Aβ. Aβ1-42 was more abundant than Aβ1-40 in this pool, and most of the insoluble Aβ1-42 was generated in the endoplasmic reticulum/intermediate compartment pathway. High levels of insoluble Aβ were also detected in several nonneuronal cell lines engineered to overexpress the amyloid-β precursor protein. This insoluble intracellular pool of Aβ was exceptionally stable, and accumulated in NT2N neurons in a time-dependent manner, increasing 12-fold over a 7-wk period in culture. These novel findings suggest that Aβ amyloidogenesis may be initiated within living neurons rather than in the extracellular space. Thus, the data presented here require a reexamination of the prevailing view about the pathogenesis of Aβ deposition in the AD brain.

Alzheimer’s disease (AD) is characterized by accumulation of fibrillar amyloid-β peptides (Aβ) in senile plaques. That the accumulation of Aβ is essential for the pathogenesis of AD is supported by genetic studies showing that mutations in the amyloid-β precursor protein (APP) (which gives rise to Aβ through proteolytic processing) are linked to a subset of familial AD (FAD) cases with autosomal penetrance, and alter Aβ production (reviewed in Selkoe, 1997). For example, the double mutation found in a Swedish FAD kindred leads to overproduction of Aβ, while other mutations alter the relative levels of the two major forms of Aβ, resulting in an increased Aβ1-42/1-40 ratio (Citron et al., 1992; Scheuner et al., 1996). Previous studies have shown that Aβ1-42 is more insoluble than the more abundant Aβ1-40, and that it is the most prevalent Aβ species found in senile plaques (Iwatsubo et al., 1994). Other FAD mutations that account for the majority of early-onset FAD cases have been linked to the Presenilin 1 (PS1) and Presenilin 2 (PS2) genes (Levy-Lahad et al., 1995; Sherrington et al., 1995). Mutations in these genes, like some of those in the APP gene, also increase the Aβ1-42/1-40 ratio (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996).

Since genetic studies have established a role for Aβ in the pathogenesis of AD, it is essential to understand how Aβ is produced from APP. For example, it has been shown that APP is cleaved by β-secretase(s) to generate the NH2 terminus of Aβ, and by γ-secretase(s) to generate the COOH terminus of Aβ (Haass et al., 1992; Shoji et al., 1992). These cleavages may occur in a variety of subcellular locations, including the endoplasmic reticulum/intermediate compartment (ER/IC; Chyung et al., 1997; Cook et al., 1997; Hartmann et al., 1997; Xu et al., 1997), the trans-Golgi network (TGN; Xu et al., 1997), and the endosomal/lysosomal system (Koo and Squazzo, 1994). Whereas Aβ produced by these pathways may be secreted (as has been shown for TGN-generated Aβ) or may remain intracellular (as has been shown for Aβ generated by the ER/IC pathway), the relative roles of intracellular and secreted Aβ in the pathogenesis of AD remain to be determined.
While numerous studies have documented that nonneuronal cells engineered to express APP secrete both Aβ1-40 and Aβ1-42, intracellular Aβ is not commonly seen in these cells (Forman et al., 1997; Xu et al., 1997). However, intracellular Aβ can be detected readily in human NT2N neurons after metabolic labeling, and its production precedes that of secreted Aβ (Wertkin et al., 1993; Turner et al., 1996). Analysis of intracellular Aβ by ELISA indicates that intracellular and secreted Aβ are composed of different ratios of Aβ1-42/1-40, with Aβ1-40 being more prevalent in secreted material (Turner et al., 1996). In addition to being produced by mechanisms with different time courses, and being composed of different proportions of Aβ1-40 and Aβ1-42, intracellular and secreted Aβ can be produced by different pathways in NT2N neurons. Recent studies have shown that Aβ1-42, but not Aβ1-40, is produced by an ER/IC pathway, and that this pathway does not contribute to the secreted pool of Aβ (Cook et al., 1997). Finally, secretion of Aβ by NT2N neurons increases with time in culture (Turner et al., 1996). An age-dependent increase in Aβ secretion by neurons in vivo may play a role in the deposition of Aβ into senile plaques in the extracellular space of the brain during normal aging and in AD, as well as in the cortex and hippocampus of transgenic mice that overexpress mutant forms of APP (Games et al., 1995; Hsiao et al., 1996).

In addition to forming insoluble extracellular plaques, Aβ may also accumulate intracellularly in an aggregated insoluble pool. For example, exogenous Aβ1-42 added to culture medium can be taken up by cells, after which it can be solubilized only by formic acid extraction (Knauer et al., 1995; Yang et al., 1995). Thus, these findings raise the possibility that endogenously produced intracellular Aβ may aggregate within neurons as well. Because formic acid is required to solubilize Aβ from senile plaques, we sought to detect the presence of insoluble Aβ within NT2N neurons and other cell lines by formic acid extraction, and found that a significant fraction of the total intracellular Aβ, particularly Aβ1-42, was retained as an insoluble pool within these cells. Further, this insoluble pool of Aβ increased 12-fold in postmitotic NT2N neurons over a period of 7 wk in culture. Since the prevailing view of amyloidogenesis in AD is that plaque formation is initiated in the extracellular space by secreted Aβ, our findings challenge this assumption by implicating the intracellular compartment as a site where Aβ may accumulate in an insoluble form.

Materials and Methods

Cell Culture

NT2 cells derived from a human embryonal carcinoma cell line (Ntera2/cLiD1) were grown and passaged as described previously (Pleasure et al., 1992; Pleasure and Lec, 1993). Cells were differentiated by two weekly retinoic acid treatments (10 μM) for 5 wk, and were replated (replate 2 cells) in the presence of mitotic inhibitors to yield nearly pure NT2N neurons (Pleasure et al., 1992). To obtain 90% pure neurons (replate 3 cells), replate 2 cells were removed enzymatically and mechanically, and were replated in 10-cm dishes (Pleasure et al., 1992). Cultures of Replate 2 or Replate 3 NT2N cells were used for experiments when they were 3–4 wk old unless otherwise indicated. CHO Pro5 cells were grown and passaged three times per week in Alpha-MEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS and penicillin/streptomycin. Baby hamster kidney (BHK-21) cells were grown and passaged three times per week in Glasgow MEM (Life Technologies, Inc.) supplemented with 10% tryptone phosphate, 5% FBS, and 0.02 M Hepes. CHO-695 cells were obtained from Dr. S.S. Sisodia, and were grown and passaged as described above for CHO Pro5 cells with the addition of 0.2 mg/ml of G418 to the culture medium.

Preparation of Semliki Forest Virus and Infection of Cultured Cells

Semliki Forest Virus (SFV) expressing wild-type APP695 (SFV-APPwt) or an APP mutant in which the third and fourth amino acids from the carboxyl terminus of APP have been changed to lysines (SFV-APPACK) were prepared and titrated as previously described (Chyung et al., 1997; Cook et al., 1997). CHO-Pro5, BHK-21, NT2, and NT2N cells were infected in serum-free medium at a multiplicity of infection of ~10. After 1 h, complete growth medium was replaced and infection was allowed to proceed for 12 h.

Metabolic Labeling, Immunoprecipitation, and Gel Electrophoresis

Cultured NT2N cells were methionine-deprived by incubation in methionine-free DMEM (Life Technologies, Inc.) for 30 min before adding [35S]methionine (500 μCi/ml in methionine-free DMEM + 5% dialyzed FBS; DuPont-NEN, Boston, MA) for a 12-h labeling period. Cells were washed twice in PBS and lysed in 600 μl RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 5 mM EDTA in TBS, pH 8.0) with a cocktail of protease inhibitors (1 μg/ml each of Pepstatin A, Leupeptin, TPCK, TLCK, STI, and 0.5 mM PMSF). After brief sonication, cell lysates were centrifuged at 40,000 g for 20 min at 4°C, and the supernatant was subjected to immunoprecipitation with 6E10 (a monoclonal antibody specific for Aβ1-17; Kim et al., 1988) as previously described (Turner et al., 1996). The remaining pellets were resuspended in 100 μl 70% formic acid and sonicated until clear. For direct extraction into formic acid, cells were scraped in 1 ml PBS, pellet centrifuged, and lysed in 100 μl of 70% formic acid with sonication. Formic acid from both directly extracted and sequentially extracted samples was removed by vacuum centrifugation for 40 min, and the resulting dry pellet was resuspended in 100 μl of 60% acetonitrile. RIPA buffer (1.9 ml) was added to each of the samples before they were subjected to immunoprecipitation with 6E10. Immunoprecipitated Aβ was resolved on a 10/16.5% step gradient Tris-Tricine gel, fixed in 10% methanol, dried, and placed on PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) plates for 72 h.

Trypsin Treatment of CHO Cells

CHO Pro5 cells were infected with SFV-APPwt for 12 h, rinsed twice in PBS, and incubated on ice for 20 min in either PBS alone, 10 μg/ml of trypsin (Life Technologies, Inc.) in PBS, or 10 μg/ml trypsin plus 0.1% Triton X-100 in an adaptation of a previously described technique (Turner et al., 1996; Chyung et al., 1997). Trypsin was then inactivated by adding 100 μg/ml soybean trypsin inhibitor. The treated cells were then washed with ice-cold PBS, scraped into PBS buffer, centrifuged at 2,000 g for 2 min, resuspended in 100 μl formic acid, sonicated, and centrifuged at 40,000 g for 20 min at 4°C. The supernatant was neutralized with 1.9 ml of 1 M Tris base and diluted 1:3 in H2O for quantification of Aβ1-40 and Aβ1-42 by sandwich-ELISA.

Lysis of Cells and Sandwich ELISA

For serial extraction in RIPA and formic acid, cells were washed twice in PBS and then lysed in 600 μl RIPA buffer and centrifuged for 20 min at 40,000 g at 4°C. Supernatant was subjected directly to sandwich ELISA, and the pellet was resuspended in 100 μl 70% formic acid with sonication until clear. Formic acid samples were then neutralized by adding 1.9 ml 1 M Tris base and diluted 1:3 in H2O before quantifying Aβ by sandwich-ELISA.

For direct extraction into formic acid, cells were scraped in PBS after washing twice with PBS. Cells were pelleted by centrifugation at 2,000 g for 2 min, and were then lysed in 100 μl formic acid. Insoluble material was pelleted by centrifugation at 40,000 g at 4°C for 20 min, and the supernatant was neutralized by adding 1.9 mg 1 M Tris base and diluted 1:3 in H2O before quantification of Aβ by sandwich-ELISA.

For extraction into PBS, cells were scraped in PBS after washing twice

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with PBS. Cells were lysed by sonication, and insoluble material was pelleted by centrifugation at 40,000 g at 4°C for 20 min, and Aβ in the soluble fraction was quantitated by sandwich-ELISA.

Sandwich-ELISA was performed as described previously using mAbs specific for different species of Aβ (Suzuki et al., 1994; Turner et al., 1996). BAN-50 (a mAb specific for the first 10 amino acids of Aβ) was used as a capturing antibody, and horse-radish peroxidase-conjugated BA-27 (a mAb specific for Aβ1-40) and horseradish peroxidase-conjugated BC-05 (a mAb specific for Aβ1-42) were used as secondary antibodies. To calibrate the sensitivity of the ELISA for detecting Aβ after formic acid extraction and neutralization, synthetic Aβ1-40 and Aβ1-42 peptides (Bachem Bioscience Inc., King of Prussia, PA) used to generate the standard curves were treated with formic acid and neutralized in the same manner as the cell lysates. Under these conditions, the sandwich ELISA had a detection limit of <1 femtomole of synthetic Aβ per sample. The BAN50, BA-27, and BC-05 mAbs were prepared and characterized as described previously (Suzuki et al., 1994).

**Cycloheximide Treatment**

For experiments involving cycloheximide treatments, NT2N cells were incubated in media containing 150 μg/ml cycloheximide for various time points up to 24 h. Cells were harvested and extracted sequentially in RIPA and formic acid as described above. Samples were then subjected to sandwich ELISA analysis.

**Western Blot Analysis of APP Levels**

RIPA-extracted cell lysates (15 μg) were solubilized in media containing 50 μl/well of 0.05% Triton X-100 using 1 ml of 10% RIPA. The samples were then incubated in media containing 150 μg/ml cycloheximide. After application of a rabbit anti-goat IgG linker, fragments of Aβ were separated in each cell type were determined by Western blotting in Triton X-100. We found that formic acid–solubilized intracellular Aβ was resistant to trypsin digestion in the absence of detergent, but sensitive to trypsin digestion after solubilization by Triton X-100 (data not shown). This finding indicates that the formic acid–soluble pool of Aβ is located intracellularly, and is accessible to trypsin only when cell membranes are first permeabilized by detergent. Finally, we found that cells extracted directly into formic acid yielded amounts of Aβ similar to the sum of RIPA-soluble and RIPA-insoluble Aβ (Fig. 1 A). From these studies, we concluded that neurons contain at least two major pools of intracellular Aβ: a detergent soluble pool, and a larger formic acid soluble pool that is enriched in Aβ1-42.

**Results**

**Neurons Contain Insoluble Amyloid β Peptide**

To evaluate the possibility that Aβ exists in multiple intracellular pools with different solubility characteristics, NT2N neurons were sequentially extracted in aqueous buffer (PBS), detergent buffer (RIPA), and then 70% formic acid. The levels of Aβ1-40 and Aβ1-42 present in each fraction were quantified by sandwich-ELISA. Previous studies have shown that nonionic detergents liberate intracellular Aβ, but not Aβ deposited in senile plaques or fibrillar Aβ formed in vitro (Selkoe et al., 1986; Burdick et al., 1992; Harigaya et al., 1995; Turner et al., 1996). However, more rigorous solubilization methods using 70% formic acid liberate Aβ from these insoluble aggregates. Sonication of cells in PBS in the absence of detergent failed to release any soluble Aβ (data not shown). By contrast, significant levels of Aβ1-40 and Aβ1-42 were solubilized by RIPA buffer. Nonetheless, RIPA buffer released only a fraction of the total intracellular Aβ since subsequent extraction of the detergent-insoluble material with 70% formic acid revealed a much larger pool of both Aβ species (Fig. 1 A). Since increased production of Aβ1-42 relative to Aβ1-40 has been associated with AD (Borchelt et al., 1993; Scheuner et al., 1996), we examined the ratios of these Aβ species in the detergent-soluble and -insoluble pools in NT2N neurons. The ratio of Aβ1-42/1-40 in the RIPA soluble pool was 1.0 ± 0.1 (Fig. 1 B), consistent with previous studies in a variety of experimental systems (Cook et al., 1997; Forman et al., 1997). However, Aβ1-42 was more abundant in the detergent-insoluble pool, with an Aβ1-42/1-40 ratio of 2.7 ± 0.3 (Fig. 1 B).

This finding is consistent with the reduced solubility of Aβ1-42 relative to Aβ1-40 in vitro, and the predominance of Aβ1-42 in insoluble deposits in the AD brain (Jarrett et al., 1993a; Iwatsubo et al., 1994).

The identification of a large and previously undetected pool of insoluble Aβ in NT2N neurons prompted us to establish precise conditions for reproducible recovery of the maximum amount of formic acid–extractable Aβ. Sonication was found to be necessary for efficient Aβ extraction, and a volume of 100 μl formic acid was found to extract Aβ optimally from cell lysates containing ~1 mg of total protein. However, longer incubation times in formic acid (up to 24 h) or high incubation temperatures (up to 37°C) did not increase Aβ recovery (data not shown). To confirm that formic acid–extracted Aβ was present in intracellular compartments and not attached to the cells or culture dish, cells were treated with trypsin in the presence or absence of 0.1% Triton X-100. We found that formic acid–solubilized intracellular Aβ was resistant to trypsin digestion in the absence of detergent, but sensitive to trypsin digestion after solubilization by Triton X-100 (data not shown). This finding indicates that the formic acid–soluble pool of Aβ is located intracellularly, and is accessible to trypsin only when cell membranes are first permeabilized by detergent. Finally, we found that cells extracted directly into formic acid yielded amounts of Aβ similar to the sum of RIPA-soluble and RIPA-insoluble Aβ (Fig. 1 A). From these studies, we concluded that neurons contain at least two major pools of intracellular Aβ: a detergent soluble pool, and a larger formic acid soluble pool that is enriched in Aβ1-42.

**Insoluble Aβ is Present in a Range of APP-Expressing Cell Types**

To determine if insoluble intracellular Aβ is present in cell types other than neurons, NT2, CHO Pro5, and BHK-21 cells were sequentially extracted with RIPA followed by formic acid, and Aβ levels were measured by sandwich-ELISA (Fig. 2). To evaluate the consequences of increased APP production on the generation of soluble and insoluble intracellular Aβ, each cell type was also infected with a recombinant SFV vector that led to the expression of high levels of APP695. Additionally, Aβ levels in stably transfected CHO cells expressing APP695 (CHO-695) were examined (Fig. 2 A). steadystate APP levels present in each cell type were determined by Western blotting in order to correlate the levels of intracellular Aβ with APP (Fig. 2 B).

In contrast to NT2N neurons, retinoic acid naïve NT2 cells did not produce significant amounts of Aβ, despite expressing nearly equivalent levels of APP (Fig. 2, A and B). This observation is consistent with previous experiments that have demonstrated that NT2 cells do not efficiently process APP by the β-secretase pathway, and thus generate only low levels of Aβ (Wertkin et al., 1993; Forman et al., 1997). Furthermore, the engineered expression of APP695 in NT2 cells at levels similar to those found in NT2N neurons resulted in only a modest increase in intracellular Aβ levels (Fig. 2, A and B), indicating that the lack of intracellular Aβ in NT2 cells relative to NT2N neurons was not due to differential expression of APP iso-
forms in the two cell types (APP751/770 in NT2 cells vs. APP 695 in NT2N cells), but to differential processing of APP. In addition, the fact that only low levels of Aβ were detected by sandwich-ELISA in this cell line further confirms that this assay is highly specific for Aβ, and does not significantly cross-react with other cellular proteins, including full-length APP, other Aβ-containing carboxy-terminal fragments, or non-Aβ APP-derived fragments.

CHO Pro5 and BHK-21 cells expressed barely detectable levels of APP, and they did not produce detectable levels of soluble or insoluble Aβ, further confirming the specificity of the Aβ ELISA. CHO-695 cells, however, did produce intracellular Aβ, 22 ± 3% of which was insoluble (Fig. 2A). Likewise, infection of CHO Pro5 cells and BHK-21 cells with SFV-APPwt led to a markedly increased production of APP as well as intracellular Aβ, of which up to 74 ± 5% was insoluble. This dramatic increase in Aβ production over a relatively short period of time could favor Aβ aggregation, resulting in a decrease in Aβ solubility. Indeed, CHO cells stably expressing APP contained a much lower proportion of insoluble Aβ than did SFV-APP–infected CHO cells (Fig. 2A).

These findings indicate that in addition to cell type–specific factors, the level of APP expression also governs deposition of insoluble Aβ. In cells that efficiently use the β-secretase pathway to generate Aβ, increased APP expression generally resulted in increased levels of both soluble and insoluble Aβ. However, while CHO-695 cells and NT2N neurons both expressed similar levels of APP and produced similar levels of soluble Aβ, NT2N neurons accumulated significantly higher levels of insoluble Aβ (Fig. 2; compare tracks labeled NT2N vs. CHO-695). This difference may be due to the higher metabolic rate of CHO-695 cells, which may result in increased turnover of Aβ, thus hindering aggregation. Alternatively, Aβ aggregation in CHO-695 cells may be impeded by continual dilution due to cell division. In postmitotic neurons, Aβ may accumulate intracellularly over time, and thus favor the formation of insoluble aggregates.

Taken together, these results indicate that while NT2N neurons accumulate intracellular insoluble Aβ as a consequence of endogenous APP production, other cell types also exhibit this property when they overexpress APP. It is interesting to note that increased expression of APP in NT2N neurons as a consequence of SFV-APPwt infection did not result in increased levels of intracellular Aβ1-42, consistent with some of our previous work indicating that γ-secretase cleavage in the ER/IC pathway is rate-limiting (Cook et al., 1997). By contrast, increased expression of APP in NT2N neurons resulted in increased levels of intracellular Aβ1-40. That this increase was due solely to increased levels of soluble Aβ1-40 is consistent with this form of Aβ being produced late in the secretory pathway, and being recovered from cells before secretion.

**Aβ Can be Immunoprecipitated from an Insoluble Pool**

To further confirm that the material recovered by extraction with formic acid and measured by sandwich ELISA was indeed Aβ, SFV-APPwt–infected NT2N and CHO cells were metabolically labeled with [35S]methionine for 22 h, and Aβ was immunoprecipitated using 6E10. As shown in Fig. 3, a band of ~4 kD was immunoprecipitated by an Aβ-specific antibody in the RIPA-soluble cell lysate. Additional Aβ was immunoprecipitated from the RIPA-insoluble (formic acid–extracted) cellular fraction, thus confirming that a pool of Aβ remained insoluble in RIPA buffer, and could be extracted by formic acid (Fig. 3). However, the yield of Aβ after formic acid extraction was lower than that predicted by Aβ sandwich ELISA. To determine if formic acid extraction compromised the recovery of Aβ by immunoprecipitation, [35S]methionine-labeled SFV-APPwt–infected cells were extracted directly into formic acid. Direct extraction of cells into formic acid would be expected to yield amounts of Aβ equal to the sum of Aβ extracted in the RIPA-soluble and -insoluble pools. However, lower levels of Aβ than expected were recovered by this method (Fig. 3; compare lane 3 with lanes 1 and 2, and lane 6 with lanes 4 and 5). Thus, immunoprecipitation of formic acid–extracted cells was not quantitative, and resulted in only partial recovery of Aβ. This low recovery of Aβ may have been due to incomplete resolubilization of Aβ in acetone/after lyophilization, or re-aggregation of Aβ during immunoprecipitation. To evaluate the contribution of each of these factors to the incomplete recovery of Aβ by immunoprecipitation, we measured Aβ levels in the formic acid–extracted cell lysate before and after lyophilization and immunoprecipitation by sandwich-ELISA. We found that ~43% of formic acid–extracted Aβ could be resolubilized in acetone/after lyophilization.
after lyophilization, and ~45% of this resolubilized Aβ could be captured by immunoprecipitation with the antibody 6E10 (data not shown). Nevertheless, despite the shortcomings of the immunoprecipitation protocol as compared with the Aβ sandwich-ELISA, these data confirm that the formic acid–extracted pool does indeed contain Aβ.

**Differential Production of Insoluble Aβ1-40 and Aβ1-42 in Subcellular Compartments**

While it has been shown that secreted Aβ is mainly produced in the TGN, intracellular Aβ1-42, but not Aβ1-40, is produced in the ER/IC (Cook et al., 1997). To determine if Aβ1-42 produced in the ER/IC enters the insoluble pool, NT2N neurons and CHO Pro5 cells were infected with SFV-APPwt or SFV-APPΔKK (an APP mutant containing the dilsysine ER retrieval sequence). Infection of both cell types with SFV-APPΔKK gave similar results: almost a complete abrogation of Aβ1-40 production, with no diminution of Aβ1-42 production relative to SFV-APPwt infected cells (Fig. 4, A and B). Importantly, the levels of insoluble Aβ1-42 were the same in SFV-APPwt and SFV-APPΔKK–infected cells. These results demonstrate that Aβ1-42 produced in the ER/IC pathway represents the bulk of the insoluble Aβ1-42 inside cells. By contrast, insoluble Aβ1-40 is produced by a post-ER/IC pathway. Finally, these results also prove that insoluble Aβ can accumulate in the absence of secretion, and they provide additional evidence that the Aβ solubilized by formic acid is intracellular.

**Time-dependent Accumulation of Insoluble Aβ**

Our previous studies have shown that secretion of Aβ1-40 and Aβ1-42 by the NT2N neurons increases with time in culture without an increase in APP synthesis (Turner et al., 1996). However, a time-dependent increase in intracellular Aβ was not detected. Conversely, we found that retention of APP in the ER/IC resulted in continued production of Aβ1-42, but without either secretion or intracellular accumulation (Cook et al., 1997). Our observation here that intracellular Aβ (particularly the Aβ1-42 species produced in the ER/IC) forms an insoluble pool provided a

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**Figure 2.** FA extraction of a variety of cell lines reveals the presence of varying levels of insoluble intracellular Aβ. (A) Uninfected and SFV-APPwt-infected NT2 cells, NT2N cells (replate 2, 4 wk old), CHO Pro5 cells, CHO-695 cells (uninfected only), and BHK-21 cells were sequentially extracted in RIPA followed by FA. Aβ 1-40 and 1-42 levels in the RIPA and FA samples were quantified by sandwich ELISA. Means and standard errors (four separate experiments done in triplicate) of Aβ levels are shown. (B) Samples from RIPA cell lysates of each of these cell lines (both uninfected and SFV-APPwt infected) were resolved on a 7.5% Tris-glycine acrylamide gel, and immunoblotted with Karen antibody; bands were detected by PhosphorImager after using an I^{125}-labeled secondary antibody.

**Figure 3.** Aβ can be recovered from RIPA-soluble and RIPA-insoluble (FA-solubilized) fractions of cell lysate. SFV-APPwt-infected NT2N cells and CHO Pro5 cells were metabolically labeled for 12 h before being lysed in either FA or RIPA, followed by extraction of insoluble material by FA. RIPA and FA samples were subjected to immunoprecipitation with 6E10, and were resolved on a 10/16.5% step gradient Tris-tricine gel. Molecular weight standards and Aβ bands are labeled.
possible explanation for both of these earlier findings. To test the hypothesis that insoluble Aβ can accumulate intracellularly over time, NT2N neurons were analyzed at various time points after replating by sequential extraction in RIPA and formic acid, followed by sandwich-ELISA for Aβ quantitation. We found a dramatic increase (12-fold over 7 wk in culture) in the levels of formic acid–extractable intracellular Aβ1-40 and Aβ1-42 in NT2N cells concomitant with increased time in culture (Fig. 5, A and B). In addition to an increase in the absolute amount of insoluble Aβ with longer times in culture, an increase in the fraction of insoluble Aβ was also observed. For example, at 4 wk, ~58% of Aβ was insoluble, while at 7 wk ~78% of Aβ was insoluble (Fig. 5, A and B). This result suggests that the equilibrium of soluble to insoluble Aβ may be shifted to favor insoluble Aβ in NT2N cells that were cultured longer (i.e., older neurons).

The Intracellular Accumulation of Aβ Over Time in Culture is Due to the Slow Turnover of Insoluble Aβ

The time-dependent accumulation of insoluble intracellular Aβ in neurons could be due to several factors, including the slow turnover of insoluble Aβ. To examine this possibility, we treated NT2N cells with cycloheximide to prevent protein synthesis, and measured endogenous levels of Aβ in the soluble and insoluble pools over time in culture. This approach was needed (rather than a standard pulse-chase analysis) because immunoprecipitation of Aβ after formic acid extraction was not quantitative (Fig. 3). Fig. 6 shows that over the 24-h cycloheximide treatment, soluble Aβ1-40 and Aβ1-42 decreased by ~63% and ~77%, respectively. Assuming a constant rate of degradation, we calculated half-lives of ~18 h and ~12 h for the decay of intracellular soluble Aβ1-40 and Aβ1-42, respectively. By contrast, insoluble Aβ1-40 and Aβ1-42 levels did not decrease significantly over 24 h. The slow turnover of the insoluble pool of Aβ precluded an accurate estimate of the half-life of this pool. In addition, this analysis is complicated by two factors. First, although no new APP will be synthesized in the presence of cycloheximide, existing pools of APP continue to be processed to generate Aβ. However, the half-life of APP in NT2N neurons is ~3 h. Thus, de novo production of Aβ from existing pools of APP is unlikely to contribute significantly to intracellular Aβ pools, especially at later time points. Second, soluble Aβ1-40 and Aβ1-42 may enter the insoluble pool over time, again making accurate estimates of turnover rates difficult. Nevertheless, our results show that intracellular insoluble Aβ is very long-lived, and that this long life is
likely to play an important role in the time-dependent accumulation of insoluble Aβ we observed in NT2N cells over weeks in culture.

Discussion

The presence of insoluble aggregates of Aβ in senile plaques is a well-characterized feature of AD (Selkoe, 1997). Aβ is composed of two major species that terminate at residues 40 and 42 of the intact Aβ sequence. Both species can be recovered from the CSF of normal and AD individuals, with Aβ1-40 being approximately 10-fold more abundant than Aβ1-42 (Citron et al., 1992). However, Aβ1-42 is the major Aβ species present in senile plaques, with Aβ1-40 being only a minor constituent (Iwatsubo et al., 1994). That alterations in APP processing can lead to development of AD has been shown by several FAD-associated APP mutations that, when expressed in vitro or in transgenic animals, lead to either an overall increase in Aβ production or an increase in the amount of Aβ1-42 relative to Aβ1-40 (Borchelt et al., 1996; Duff et al., 1996). The differential production of Aβ1-40 and Aβ1-42 as a consequence of AD-associated APP mutations as well as the preferential deposition of Aβ1-42 in senile plaques raises important questions as to the intracellular sites of Aβ1-42 generation, the origin of Aβ that is recovered from senile plaques, and the factors that control its deposition.

Both Aβ1-40 and Aβ1-42 are constitutively produced and secreted from cells in vitro and in vivo as judged by their recovery from conditioned medium and CSF (Shoji et al., 1992; Tamaoka et al., 1996). Since FAD-associated APP mutations lead to increased secretion of Aβ, and senile plaques are extracellular lesions, it is possible that secreted Aβ is ultimately deposited in senile plaques, even though the factors controlling its deposition are obscure. However, we have recently discovered that retention of APP in the ER/IC induced by a variety of methods leads to continued production of intracellular Aβ1-42, but not Aβ1-40 (Cook et al., 1997). While Aβ1-42 is constitutively produced by this novel pathway in NT2N neurons, other cell types can also process APP to generate Aβ in the ER/IC after overexpression of APP (Wild-Bode et al., 1997). Aβ1-42 has also been shown to be localized to the ER/IC by immunoelectron microscopy and by cell fractionation (Hartmann et al., 1997; Wild-Bode et al., 1997). Interestingly, this compartment also is the site where PS1 and PS2 are localized (Cook et al., 1996; Kovacs et al., 1996). Since mutations in PS1 and PS2 account for the majority of early-onset FAD cases, and FAD-associated PS1 and PS2 mutations have been shown to result in an increased ratio of Aβ1-42/1-40 (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996), colocalization of the presenilins with a major site of constitutive Aβ1-42 production raises the possibility that alterations in Aβ production by the ER/IC pathway may play an important role in AD pathogenesis.

While retention of APP in the ER/IC resulted in continued and selective production of Aβ1-42, we were unable to document either secretion of this material or its intracellular accumulation (Cook et al., 1997). Taken at face value, this result indicates that the production and turnover of Aβ1-42 by the ER/IC pathway are in equilibrium. However, given the propensity of Aβ1-42 to aggregate in vivo and in vitro, we asked whether Aβ1-42 also aggregated intracellularly. Since formic acid has been shown to effectively solubilize aggregated Aβ present in senile plaques, we solubilized cell lysates in formic acid. Using this approach, we found that a considerable fraction of total intracellular Aβ1-42, and to a lesser extent Aβ1-40, could be solubilized by formic acid, but not by a variety of detergents. Currently, we do not know whether or not the formic acid–extractable Aβ self-aggregates or coaggregates with other proteins. Our observation that none of the cell-associated Aβ (including that targeted for secretion) can be extracted with aqueous buffer suggests that it may be bound to other cellular proteins. On the other hand, in vitro studies of Aβ aggregation suggest that Aβ is prone to self-aggregation. Future ultrastructural studies on the accumulated intracellular Aβ will help to resolve this issue. Intracellular insoluble Aβ was recovered in a number of different APP-expressing cell lines. Overexpression of APP generally resulted in increased production of insoluble Aβ. However, insoluble Aβ was produced most efficiently...
in NT2N neurons. Thus, while aggregation of intracellular Aβ is not cell type–specific, the subcellular environment in neurons appears to favor this process.

Identification of a novel form of intracellular Aβ that has previously escaped detection could explain our failure to detect secretion or intracellular accumulation of Aβ1-42 produced by the ER/IC pathway (Cook et al., 1997). To test this possibility, we expressed APP bearing an ER/IC retrieval signal in the cytoplasmic domain in NT2N neurons and in CHO cells. Production of intracellular soluble and insoluble Aβ1-40 was almost completely inhibited after expression of this construct, while levels of soluble and insoluble intracellular Aβ1-42 were unchanged by ER retention. Thus, almost all of the formic acid–soluble Aβ1-42 can be derived from the ER/IC pathway. By extension, insoluble Aβ1-40 must be produced by a post-ER/IC compartment. Production of insoluble Aβ1-40 and Aβ1-42 in different subcellular compartments may help explain the predominance of Aβ1-42 in the intracellular pool. Aβ1-40 is produced late in the biosynthetic pathway, and may spend relatively little time in the cell before secretion, thereby minimizing the opportunity for aggregation. By contrast, the bulk of the intracellular Aβ1-42 is produced by the ER/IC pathway. This fact represents an environment distinct from that in which Aβ1-40 is produced, and one that does not result in Aβ1-42 secretion. The long-lived nature of Aβ1-42, its continued production at an intracellular site from which it cannot be secreted, and the fact that it is intrinsically less soluble than Aβ1-40 all may contribute to its propensity to enter a stable, intracellular pool of insoluble material. It will be important to define further the factors that govern Aβ deposition in this insoluble pool, and to more carefully study its physical state.

During the course of our experiments, we found that recovery of insoluble Aβ from NT2N neurons was somewhat variable. However, we found that this result was due to a time-dependent accumulation of Aβ. Specifically, we found that Aβ levels increased by 12-fold as the NT2N neurons aged over 7 wk in culture. While insoluble Aβ1-40 and Aβ1-42 accumulated at similar rates, more detailed kinetic studies are needed to determine if production of insoluble Aβ1-40 and Aβ1-42 is contemporaneous, or if generation of insoluble Aβ1-42 seeds subsequent polymerization of Aβ1-40, as has been reported in vitro. In any event, time-dependent accumulation of insoluble Aβ could be due to increased production, decreased turnover, or stable accumulation of Aβ at a relatively constant rate. We found that intracellular insoluble Aβ was exceptionally stable. Thus, even slow addition of Aβ to the insoluble pool over weeks in culture could result in steady accumulation of Aβ seen in the insoluble pool over time. This observation may have implications for AD pathogenesis, where it is thought that accumulation of Aβ occurs slowly over decades. Since AD is an age-dependent disease, the data presented here suggest that gradual accumulation of intracellular Aβ may be a factor in the slow onset and progression of AD. It will be important to determine if accumulation of intracellular insoluble Aβ is simply the result of the stability of this form of Aβ, or if other time-dependent factors (such as altered APP processing or neurotoxic insults) contribute to this process.

Although intracellular β-amyloid fibrils have been observed in the AD brain (Kim et al., 1988) as well as in a transgenic mouse model of AD (Masliah et al., 1996), it is unclear whether Aβ fibrils can form within neurons from endogenously produced Aβ. The experiments presented here demonstrate that significant levels of Aβ are insoluble within neurons. The observation that Aβ can accumulate with time in a relatively stable insoluble pool may explain how Aβ deposition in senile plaques can begin despite relatively low levels of secreted and CSF-soluble Aβ1-42. Concentrated intracellular Aβ1-42 could rapidly nucleate fibril formation, and intracellularly produced Aβ1-42 and Aβ1-40 could add to these fibrils over time, thus serving as a nidus for a developing senile plaque.

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