Amyloid β-Protein Aggregation Nullifies Its Pathologic Properties in Cultured Cerebrovascular Smooth Muscle Cells*

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Judianne Davis-Salinas and William E. Van Nostrand†
From the Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717-4025

Alzheimer’s disease and related disorders are characterized by deposition of aggregated amyloid β-protein (Aβ) and accompanying pathologic changes in the neuropil and in the walls of cerebral blood vessels. Aβ induces neurotoxicity in vitro, and this effect is markedly enhanced when the peptide is preaggregated. Recently, we reported that freshly solubilized Aβ1–42 can induce cellular degeneration and a striking increase in the levels of cellular amyloid β-protein precursor and soluble Aβ peptide in cultured cerebrovascular smooth muscle cells (Davis-Salinas, J., Saporito-Irwin, S. M., Cotman, C. W., and Van Nostrand, W. E. (1995) J. Neurochem. 65, 931-934). In the present study, we show that preaggregation of Aβ1–42 abolishes the ability of the peptide to induce these cellular pathologic responses in these cells in vitro. These findings suggest that distinct mechanisms for Aβ-induced cytotoxicity exist for cultured neurons and cerebrovascular smooth muscle cells, supporting that different processes may be involved in the parenchymal and cerebrovascular pathology of Alzheimer’s disease and related disorders.

Alzheimer’s disease (AD) is characterized by deposition of the 39–42-amino acid amyloid β-protein (Aβ) in senile plaques within the neuropil and in the walls of cerebral blood vessels (1–4). Similar pathologic Aβ deposits are observed in patients with Down’s syndrome, hereditary cerebral hemorrhage with amyloidosis Dutch-type, and, to a lesser extent, in normal aging (2, 4, 5). Aβ is proteolytically derived from its transmembrane parent molecule, the amyloid β-protein precursor (AβPP) (6–9). AβPP arises from alternative splicing of mRNA encoded by a single gene located on chromosome 21, yielding primarily proteins of 695, 751, and 770 amino acids (10–12). The 751 and 770 isoform contain an additional 56-amino acid domain that shows homology to Kunitz-type serine protease inhibitors and were shown to be analogous to the cell-secreted protease inhibitor protease nexin-2 (PN-2) (13, 14). Normal secretion of AβPP involves proteolytic cleavage through the Aβ domain, thus precluding the formation of intact Aβ (15, 16). Therefore, Aβ must arise through an alternative processing pathway, possibly involving amyloidogenic intermediates generated in an intracellular endosomal/lysosomal compartment (17, 18). Recent studies have shown that extracellular, soluble Aβ is a product of normal cellular catabolism of AβPP and can be found in the medium of cultured cells and in biological fluids (19–21). Secreted PN-2/AβPP and soluble extracellular Aβ are normally expressed in many cell types of the brain. However, through an undetermined mechanism the soluble Aβ peptide becomes insoluble, aggregated, and deposited in senile plaques and within the walls of the cerebrovasculature.

The deposition of Aβ within the walls of cerebral blood vessels is a pathologic trait often seen in patients with AD. Aβ1–39, Aβ1–40, and Aβ1–42 have all been reported to be constituents of cerebrovascular amyloid (22–25). Several findings have implicated smooth muscle cells as participants in the pathology and production of AβPP and Aβ in the cerebrovasculature. For example, immunohistochemical and ultrastructural studies have shown that deposition of Aβ in the walls of the cerebral blood vessels is accompanied by extensive degeneration of the smooth muscle cells, suggesting a toxic effect of the amyloid on these cells in vivo (26–29). Immunohistochemical studies have implicated the smooth muscle cells in the production of AβPP and Aβ in the cerebrovasculature (27–30). In addition, cerebrovascular smooth muscle cells have been shown to synthesize AβPP and produce extracellular, soluble Aβ in culture (31, 32).

Recently, we described the effects of synthetic Aβ peptides on primary cultured human leptomeningeal smooth muscle (HLSM) cells. Incubation of Aβ1–42 with HLSM cells caused extensive cellular degeneration accompanied by striking increases in the levels of cellular AβPP and extracellular, soluble Aβ peptide (32). However, the effects seen with Aβ1–42 were not observed when HLSM cells were incubated with the shorter Aβ1–39 and Aβ1–40 isoforms, suggesting that the longer Aβ peptide is the pathologic isofrom in the cerebrovasculature. These data suggested a novel product-precursor mechanism which could result in the adverse production and accumulation of potentially amyloidogenic Aβ fragments and the spread of the cerebrovascular pathology. Since extracellular, soluble Aβ peptide is a normal product of cellular metabolism (19–21), this creates the paradox as to how Aβ could contribute to the cellular pathology of AD and related disorders.

Previous studies have reported that the application of Aβ peptides to primary cultures of rat and human embryo cortical and hippocampal neuronal cells will cause toxicity (33, 34). Aβ peptides can exist in both soluble and insoluble, aggregated forms. Subsequent studies have shown that the neurotoxicity observed in vitro appears to reside in the insoluble, aggregated forms of Aβ (35, 36). Since aggregated forms of Aβ are also observed in the cerebrovasculature of patients with AD and related disorders (1–5), we investigated the ability of preaggre-
gated $\text{A} \beta_{1-42}$ to elicit the cellular pathologic changes in cultured HLSM cells.

**EXPERIMENTAL PROCEDURES**

$\text{A} \beta_{1-42}$ Peptides—$\text{A} \beta_{1-42}$ was synthesized and characterized as described by Burdick et al. (37). The lyophilized peptide was resuspended in sterile water to a concentration of 0.25 mM. The $\text{A} \beta_{1-42}$ peptide was subjected to several cycles of freezing and incubating at 37°C to promote aggregation (36). The freshly solubilized and aggregated forms of the $\text{A} \beta_{1-42}$ peptide were characterized by light microscopy and by turbidity spectrophotometric measurements at 400 nm as described (38).

Cell Culture—Primary cultures of HLSM cells were established and characterized as described previously (31). The cultures were maintained in 12-well culture dishes with Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Life Technologies, Inc.), 20 ng/ml insulin-like growth factor-I, 1 µg/ml hydrocortisone, and antibiotics.

For experiments, the near-confluent cultures of HLSM cells were placed in serum-free medium containing 0.1% bovine serum albumin for 24 h prior to treatment. Freshly solubilized or preaggregated $\text{A} \beta_{1-42}$ was added to the cultures in fresh serum-free medium and incubated at 37°C for various lengths of time. Cells were routinely viewed and photographed using an Olympus phase-contrast microscope. Cell viability was quantitated using a fluorescent live/dead cell assay (Molecular Probes) as described by the manufacturer. The cultures were viewed under a Nikon fluorescence microscope, and the number of live and dead cells were scored from at least three separate wells for each sample.

**Immunoblot Analysis of $\text{A} \beta_{1-42}$**—HLSM cells were incubated for 6 days in the presence of 25 µM freshly solubilized or an equivalent amount of preaggregated $\text{A} \beta_{1-42}$; the medium was collected and the cells rinsed with phosphate-buffered saline. The cells were then solubilized in a buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 1% SDS, 5 mM EDTA, 500 µM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 µM leupeptin, and 10 µM chymostatin. The cell lysates were centrifuged at 14,000 × g for 10 min to remove insoluble material. The protein concentrations of the resulting supernatants were determined by the method of Bradford (39). The culture medium and lysate samples were stored at −30°C until analysis. Aliquots of cell lysate and culture medium samples were electrophoresed on nonreducing SDS-10% polyacrylamide gel, and the proteins were transferred to nitrocellulose. The membranes were then probed with monoclonal antibody P2-1 (5 µg/ml), which specifically recognizes an epitope near the amino terminus of $\text{A} \beta_{1-42}$ and then incubated with a secondary peroxidase-coupled sheep anti-mouse IgG antibody at a 1:700 dilution. The peroxidase activity on the membranes was detected using an enhanced chemiluminescence system (Amersham), and the blots were exposed to Kodak X-Omat AR film. The absolute levels of secreted and cellular $\text{A} \beta_{1-42}$ were quantitated from the immunoblots by scanning laser densitometry and comparison with scans of standard curve immunoblots containing known amounts of purified $\text{A} \beta_{1-42}$.

**RESULTS AND DISCUSSION**

Senile plaques found in brains of patients with AD and related disorders are composed of insoluble $\text{A} \beta$ aggregates (3, 4). Previous studies have shown that incubation of preaggregated synthetic $\text{A} \beta$ peptides with primary rat or human cortical and hippocampal neuronal cultures causes enhanced neurotoxicity compared with freshly solubilized $\text{A} \beta$ peptides (33-36). These findings suggest that $\text{A} \beta$ aggregation is an important contributing factor to neuronal degeneration and toxicity. The amyloid deposits found in the cerebrovasculature of patients with AD and related disorders also contain aggregated forms of $\text{A} \beta$ and that are associated with degenerating smooth muscle cells in the vessel walls (23-30). Since aggregation of $\text{A} \beta$ is important in causing neurotoxicity, in the present study we investigated the effects of aggregated $\text{A} \beta_{1-42}$ on the cellular degeneration of cerebrovascular smooth muscle cells in vitro.

Synthetic $\text{A} \beta_{1-42}$ peptide was preaggregated as described by Pike et al. (35, 36). The extent of aggregation was quantitatively assessed by spectrophotometric turbidity measurements (38). As shown in Fig. 1A, the preaggregated $\text{A} \beta_{1-42}$ exhibited a pronounced optical density compared with the freshly solubilized peptide indicative of aggregates. Similarly, light microscopic analysis of the preparation showed large $\text{A} \beta_{1-42}$ aggregates (Fig. 1B) that were absent in preparations of freshly solubilized peptide (data not shown).

We performed studies to determine if the preaggregated form of $\text{A} \beta_{1-42}$ could induce cellular degeneration in the cultured HLSM cells. The cells were incubated for 6 days in the absence or presence of 25 µM freshly solubilized $\text{A} \beta_{1-42}$ or an equivalent amount of $\text{A} \beta_{1-42}$ that was preaggregated and then the cells were characterized by light microscopy. HLSM cells incubated with the freshly solubilized $\text{A} \beta_{1-42}$ (Fig. 2B) showed signs of extensive morphological degeneration compared with the untreated cells (Fig. 2A) as recently described (32). Surprisingly, HLSM cells incubated with the preaggregated $\text{A} \beta_{1-42}$ exhibited no signs of degeneration (Fig. 2C). In parallel studies the viability of the untreated and treated HLSM cells was quantitated using a fluorescent live/dead cell assay as described under "Experimental Procedures." As shown in Fig. 2D, HLSM cells incubated with the preaggregated $\text{A} \beta_{1-42}$ peptide showed no loss of viability similar to the untreated cells. However, the HLSM cells incubated with the freshly solubilized $\text{A} \beta_{1-42}$ peptide showed a ∼60% loss in cell viability. It is noteworthy that incubation of the same preparations of preaggregated and freshly solubilized $\text{A} \beta_{1-42}$ peptides with primary rat neuronal cultures produced opposite effects to those seen in the HLSM cells; preaggregated $\text{A} \beta_{1-42}$ caused a pronounced increase in neurotoxicity compared with the freshly solubilized peptide (data not shown). Together, these studies suggest that preaggregated $\text{A} \beta_{1-42}$ does not induce cytotoxicity in cultured HLSM cells. On the other hand, preaggregation of the $\text{A} \beta_{1-42}$ is an important contributing factor to eliciting neurotoxicity (35, 36). This disparity suggests that different mechanisms are involved in $\text{A} \beta$-induced toxicity in cultured neurons and cerebrovascular smooth muscle cells.

We recently reported that incubation of freshly solubilized $\text{A} \beta_{1-42}$ with cultured HLSM cells caused a striking increase in the levels of cellular $\text{A} \beta_{1-42}$ which coincided with the cellular degeneration (32). Therefore, we determined if similar in-
levels of secreted Aβ could induce several cellular pathologic responses in cultured HLSM cells. The present studies, however, demonstrate that preaggregated Aβ₁₋₄₂ is incapable of inducing these pathologic responses in the cultured HLSM cells. These findings suggest the possibility that soluble, unaggregated Aβ₁₋₄₂ may interact with a “receptor” or some other molecule on the surface of HLSM cells to initiate the molecular cascades involved with the observed pathologic responses. In this scenario, preaggregated Aβ₁₋₄₂ may be incapable of interacting with this cell surface component to initiate the pathologic responses. Alternatively, soluble Aβ₁₋₄₂ may be required to assemble into an aggregated structure on the surface of the HLSM cells in a manner that is different than the structure of aggregates that assemble in solution in the absence of cells. Regardless, the present findings indicate that preaggregated forms of Aβ are not cytotoxic to HLSM cells, whereas they have pronounced toxicity in neuronal cultures. This suggests the intriguing notion that different mechanisms of Aβ cytotoxicity exist for distinct cell types that are associated with the neuronal or cerebrovascular pathologies of AD and related disorders.

REFERENCES
Cytotoxic Properties of Aggregated Aβ


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