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Enhanced pathologic properties of Dutch-type mutant amyloid β-protein

(cerebral amyloid angiopathy/smooth muscle cells)

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ABSTRACT **Cerebrovascular amyloid β-protein (Aβ) deposition is a pathological feature of several related disorders including Alzheimer disease and hereditary cerebral hemorrhage with amyloidosis Dutch-type (HCHWA-D). HCHWA-D is caused by a point mutation in the gene that encodes the Aβ precursor and results in a Glu → Gln substitution at position 22 of Aβ. In comparison to Alzheimer disease, the cerebrovascular Aβ deposition in HCHWA-D is generally more severe, often resulting in intracerebral hemorrhage when patients reach 50 years of age. We recently reported that $A\beta_{1-42}$, but not the shorter $A\beta_{1-40}$, induces pathologic responses in cultured human leptomeningeal smooth muscle cells including cellular degeneration that is accompanied by a marked increase in the levels of cellular Aβ precursor and soluble Aβ peptide. In the present study, we show that the HCHWA-D mutation converts the normally nonpathologic $A\beta_{1-40}$ into a highly pathologic form of the peptide for cultured human leptomeningeal smooth muscle cells. These findings suggest that these altered functional properties of HCHWA-D mutated Aβ may contribute to the early and often severe cerebrovascular pathology that is the hallmark of this disorder.**

Cerebral amyloid angiopathy, a common pathological feature of patients with Alzheimer disease (AD), is characterized by deposition of the 39- to 42-amino acid amyloid β-protein (Aβ) in the media of primarily small and medium-sized arteries and arterioles of the cerebral cortex and leptomeninges (1–4). Extensive cerebral amyloid angiopathy is also the hallmark of individuals with a rare autosomal dominant disorder known as hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) (5–8). In fact this condition is usually so severe in patients with HCHWA-D that they develop recurrent and often fatal intracerebral hemorrhages at a age of 50 ± 5.6 years (mean ± SD; range = 39–71 years) (8). The Aβ that is deposited in the cerebral blood vessels is proteolytically derived from a set of larger precursor proteins, termed the Aβ precursors (AβPP) (9–11). Individuals with HCHWA-D have been shown to possess a point mutation in their AβPP gene that results in a Glu → Gln substitution at position 22 in the Aβ domain (12). Why this mutation in AβPP causes the distinctive cerebrovascular pathology of HCHWA-D remains unclear.

Cerebrovascular smooth muscle cells are intimately associated with the pathology of cerebral amyloid angiopathy. For example, deposition of Aβ in the walls of the cerebral blood vessels is accompanied by extensive degeneration of the smooth muscle cells, suggesting that amyloid has a toxic effect on these cells *in vivo* (13–15). Smooth muscle cells have been implicated in the production of AβPP and Aβ in the cerebral vasculature of patients with AD and patients with HCHWA-D (14–16). Moreover, cerebrovascular smooth muscle cells synthesize AβPP and produce extracellular soluble Aβ in culture (17–20).

Recently, we reported that $A\beta_{1-42}$ causes extensive cellular degeneration that is accompanied by a striking increase in the levels of cellular AβPP and extracellular soluble Aβ peptide in cultured human leptomeningeal smooth muscle (HLSM) cells (18, 19). However, the effects induced by $A\beta_{1-42}$ were not observed when HLSM cells were incubated with the shorter $A\beta_{1-40}$ isoform. This suggests that the longer Aβ peptide is a key pathologic isoform in the cerebral vasculature. These previous findings suggested a novel product–precursor mechanism that could result in the adverse production and accumulation of potentially amyloidogenic Aβ fragments, thereby, contributing to the spread of the pathology in patients with cerebral amyloid angiopathy. In the present study, we show that HCHWA-D mutant Aβ causes markedly enhanced pathologic responses in cultured HLSM cells. This functional alteration in Aβ may help to explain the distinctive pathology associated with HCHWA-D.

MATERIALS AND METHODS

Materials. Normal Aβ peptides and HCHWA-D Aβ peptides (Glu → Gln substitution at position 22) were synthesized and structurally characterized as described (21). Several separate syntheses of each Aβ peptide were used in the present experiments. The anti-AβPP mouse monoclonal antibody P2-1, which specifically recognizes an N-terminal epitope on all isoforms of AβPP, was prepared as described (22). Peroxidase-coupled secondary antibodies and enhanced chemiluminescence reagents were obtained from Amersham.

Cell Culture. Primary cultures of HLSM cells used in the present studies were established from leptomeningeal blood vessels obtained at autopsy and characterized as described (17). The cultures were maintained in 12-well culture dishes with Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum (GIBCO/BRL), insulin-like growth factor I (20 ng/ml), hydrocortisone (1 μg/ml), 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 Aβ peptides were added once to the cultures in fresh serum-free medium and incubated at 37°C for various periods. Cells were routinely viewed and photographed by using an Olympus phase-contrast microscope. Cell viability was quantitated by using a fluorescent live/dead cell assay (Molecular Probes) as described by the manufacturer. The

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Abbreviations: HCHWA-D, hereditary cerebral hemorrhage with amyloidosis-Dutch type; AD, Alzheimer disease; Aβ, amyloid β-protein; AβPP, amyloid β-protein precursor; HLSM cell, human leptomeningeal smooth muscle cell.

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cultures were viewed with an Olympus fluorescence microscope, and the number of live and dead cells were scored from at least three wells for each sample. For measurement of Aβ aggregation, HLSM cells were incubated with freshly solubilized Aβ peptides at 25 μM in phenol red-free serum-free DMEM. At various times, 100 μl of the cultured medium was removed from the HLSM cells and the turbidity of the samples was measured in a microtiter plate reader at 400 nm as described (19, 23).

Immunoblot Analysis of AβPP. HLSM cells were incubated for 6 days in the presence of various concentrations of freshly solubilized Aβ peptides, the medium was collected, and the cells were rinsed with phosphate-buffered saline. The cells were then solubilized in 50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% SDS/5 mM EDTA/500 μM 4-[2-aminoethyl]benzenesulfonyl fluoride/leupeptin (10 μg/ml)/chymostatin (10 μg/ml). 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 (24). The culture medium and lysate samples were stored at −30°C until analysis. Aliquots of cell lysate and culture medium samples were subjected to electrophoresis on a nonreducing SDS/10% polyacrylamide gel and the proteins were transferred to a Hybond nitrocellulose membrane (Amersham). Unoccupied sites on the membranes were blocked with 5% (vol/vol) nonfat milk. The membranes were then probed with monoclonal antibody P2-1 (5 μg/ml), which specifically recognizes an epitope near the N terminus of AβPP, and then incubated with a secondary peroxidase-coupled sheep anti-mouse IgG antibody at a 1:700 dilution. The peroxidase activity on the membrane was detected by using an enhanced chemiluminescence system (Amersham) and the blots were exposed to Kodak X-Omat AR film. The absolute levels of secreted and cellular AβPP were quantitated from the immunoblots by scanning laser densitometry and comparison to scans of standard curve immunoblots containing known amounts of purified AβPP.

RESULTS

Recently, we have reported (18) that $A\beta_{1\text{-}42}$, but not the shorter $A\beta_{1\text{-}40}$, causes extensive cellular degeneration accompanied by increased levels of cellular AβPP and soluble Aβ peptide in cultured HLSM cells. Since the cerebral amyloid angiopathy in patients with HCHWA-D generally occurs earlier and is often more severe than in patients with AD (8), we compared the effects of normal and mutant HCHWA-D synthetic Aβ peptides on the degeneration of cultured HLSM cells. HLSM cells were incubated for 6 days in the absence or in the presence of the various normal and HCHWA-D mutant Aβ peptides (Fig. 1). Consistent with our previous findings, HLSM cells incubated with normal $A\beta_{1\text{-}42}$ exhibited signs of degeneration (Fig. 1*E*), whereas cells incubated with normal $A\beta_{1\text{-}40}$ appeared unaffected (Fig. 1*C*). In contrast, HLSM cells incubated with HCHWA-D $A\beta_{1\text{-}40}$ showed signs of extensive degeneration (Fig. 1*B*), whereas cells incubated with HCHWA-D $A\beta_{1\text{-}42}$ appeared unaffected (Fig. 1*D*) similar to the untreated cells (Fig. 1*A*). These findings suggest that the HCHWA-D mutation differentially affects the ability of the Aβ peptides with different lengths to induce HLSM cellular degeneration.

HLSM cells continuously incubated with 25 μM normal $A\beta_{1\text{-}42}$ exhibited some cell death at ≈6 days, which gradually increased through 18 days with an ≈25% loss in cell viability (Fig. 2). In parallel experiments, HLSM cells incubated with 25 μM HCHWA-D $A\beta_{1\text{-}40}$ first showed cell death also at ≈6 days. However, the extent of cell death sharply increased through 12 days and by 18 days there was an ≈90% loss in cell viability. On the other hand, HLSM cells incubated in the absence of peptide or in the presence of 25 μM normal $A\beta_{1\text{-}40}$ or 25 μM HCHWA-D $A\beta_{1\text{-}42}$ showed no loss of cell viability even after >18 days of incubation. These data indicate that HCHWA-D mutant $A\beta_{1\text{-}40}$ induces pronounced toxicity to cultured HLSM cells compared to normal $A\beta_{1\text{-}42}$.

We have shown (18, 19) that HLSM cells treated with $A\beta_{1\text{-}42}$ caused a striking increase in the levels of cellular AβPP. Therefore, we compared the effects that the normal and HCHWA-D Aβ peptides had on the levels of secreted and

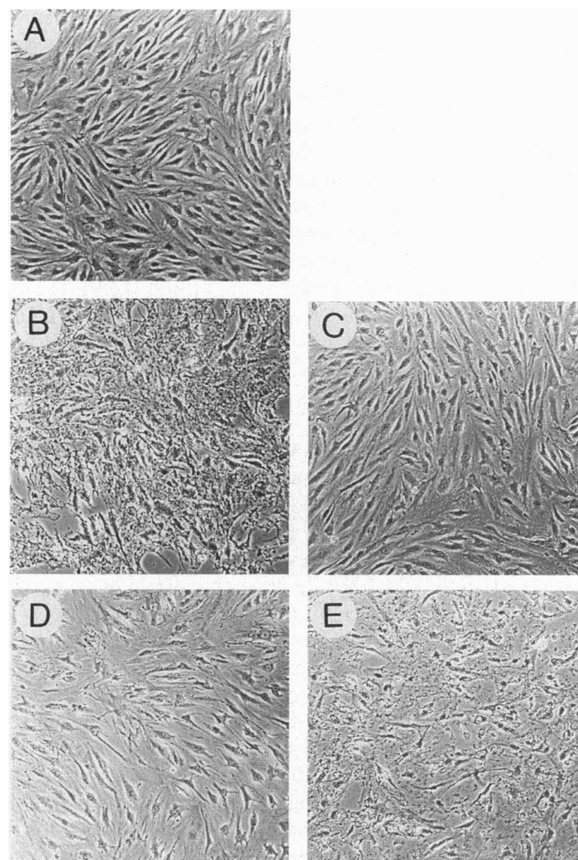

FIG. 1. Normal and HCHWA-D Aβ peptides induce cellular degeneration in cultured primary HLSM cells. Primary cultures of HLSM cells were incubated with Aβ peptides for 6 days and then photographed by using phase-contrast microscopy. (*A*) Control. (*B*) HCHWA-D $A\beta_{1\text{-}40}$ (25 μM). (*C*) Normal $A\beta_{1\text{-}40}$ (25 μM). (*D*) HCHWA-D $A\beta_{1\text{-}42}$ (25 μM). (*E*) Normal $A\beta_{1\text{-}42}$ (25 μM). (×25.)

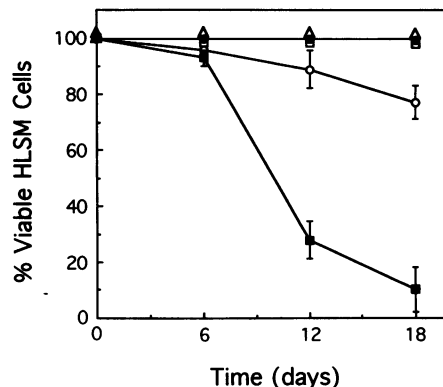

FIG. 2. Toxicity of normal and HCHWA-D Aβ peptides for cultured HLSM cells. Cultures of HLSM cells were incubated in the absence of peptide (△) or in the presence of 25 μM HCHWA-D $A\beta_{1\text{-}40}$ (■), 25 μM normal $A\beta_{1\text{-}40}$ (□), 25 μM HCHWA-D $A\beta_{1\text{-}42}$ (●), or 25 μM normal $A\beta_{1\text{-}42}$ (○) for various lengths of time, and viability of the cells was assessed by using a fluorescent live/dead cell assay. Each point represents the mean ± SD of four or more determinations.

cellular AβPP. Representative immunoblots of the secreted and cellular AβPP are shown in Fig. 3 *A* and *B*, respectively, and quantitation of the AβPP levels is shown in Fig. 3 *C* and *D*. Incubation of the HLSM cells for 6 days with 25 μM HCHWA-D $A\beta_{1\text{-}40}$ caused a >4-fold increase in the levels of secreted AβPP compared to the untreated cells (Fig. 3 *A* and *C*). On the other hand, normal $A\beta_{1\text{-}40}$, normal $A\beta_{1\text{-}42}$, or HCHWA-D $A\beta_{1\text{-}42}$ did not have an appreciable effect on the levels of secreted AβPP in cultured HLSM cells. Incubation of the HLSM cells with 25 μM normal $A\beta_{1\text{-}42}$ for 6 days caused a >10-fold increase in the amount of cellular AβPP (Fig. 3 *B* and *D*) consistent with our previous findings (18, 19). Interestingly, a more robust effect was observed when HLSM cells were incubated with 25 μM HCHWA-D $A\beta_{1\text{-}40}$ resulting in an ≈25-fold increase in the levels of cellular AβPP. In contrast, normal $A\beta_{1\text{-}40}$ and HCHWA-D $A\beta_{1\text{-}42}$ had no appreciable effect on the levels of HLSM cellular AβPP. To further compare the effects of the normal and HCHWA-D mutant Aβ isoforms on the HLSM cellular AβPP levels, the cells were incubated with increasing concentrations of the different peptides for 6 days. Elevated levels of cellular AβPP were first observed at ≈10 μM peptide and the effect was maximal between 25 and 50 μM peptide for both normal $A\beta_{1\text{-}42}$ and HCHWA-D $A\beta_{1\text{-}40}$ (Fig. 4). However, no appreciable effect on HLSM cellular AβPP levels was observed when the cells were incubated with normal $A\beta_{1\text{-}40}$ or HCHWA-D $A\beta_{1\text{-}42}$ over the concentration ranges examined. Time course studies using 25 μM HCHWA-D $A\beta_{1\text{-}40}$ showed that the levels of HLSM cellular AβPP increased steadily through 6–9 days and then decreased with increasing levels of cell death (data not presented). Thus, these findings suggest that HCHWA-D $A\beta_{1\text{-}40}$ induces much more robust effects on the levels of secreted and cellular AβPP in HLSM cells than does normal $A\beta_{1\text{-}42}$. On the other hand, similar to normal $A\beta_{1\text{-}40}$, HCHWA-D $A\beta_{1\text{-}42}$ was incapable of inducing secreted or cellular AβPP levels in the cultured HLSM cells.

It has been reported that synthetic Aβ peptides containing the HCHWA-D mutation at position 22 aggregate much more rapidly than normal Aβ peptides (25, 26). In light of this report, we compared the rate and extent of aggregation of the normal and HCHWA-D Aβ peptides in the presence of the cultured HLSM cells. HCHWA-D $A\beta_{1\text{-}42}$ was found to extensively aggregate in the culture medium within a 1-h incubation in the presence of the HLSM cells (Fig. 5). The presence of these aggregates was visible at the light microscopic level (data not presented). In contrast, little, if any, aggregation was observed in the culture medium even after a 6-day incubation of normal $A\beta_{1\text{-}40}$, normal $A\beta_{1\text{-}42}$, or HCHWA-D $A\beta_{1\text{-}40}$ with the HLSM cells (Fig. 5). These findings suggest that the presence of the Glu → Gln substitution in HCHWA-D $A\beta_{1\text{-}42}$ leads to rapid aggregation of this peptide in the presence of the HLSM cells but this effect is not observed with the shorter HCHWA-D $A\beta_{1\text{-}40}$ or the corresponding normal Aβ peptides at the concentrations examined.

DISCUSSION

Although cerebral amyloid angiopathy is a hallmark of both AD and HCHWA-D, this condition is generally more severe in patients afflicted with this latter disorder (1–8). In fact, we have found that the levels of Aβ present in isolated intracortical and leptomeningeal microvessels from patients with HCHWA-D were consistently >10-fold higher than microvessels from AD patients neuropathologically characterized to have extensive cerebral amyloid angiopathy (data not presented). Another distinction is that patients with HCHWA-D do not develop the mature senile plaques and neuronal degeneration that are typical of patients with AD (27–29). These differences in pathologic presentation of HCHWA-D and AD raise the intriguing question as to how the Glu → Gln substitution at position 22 in HCHWA-D Aβ promotes the extensive cerebral amyloid angiopathy characteristic of this disorder. One possibility might be that the presence of the mutation in HCHWA-D could lead to altered proteolytic processing of AβPP resulting in the formation of excessive amounts of Aβ. However, studies showed that the proteolytic processing of AβPP was not affected in cultured cells transfected to overexpress HCHWA-D-mutated AβPP (30). Alternatively, it was reported that synthetic Aβ peptides containing the HCHWA-D mutation aggregated much more rapidly than normal Aβ peptides (25, 26). This suggests that this mutation could cause the characteristic pathology of HCHWA-D by promoting amyloid fibril formation from soluble Aβ peptide (26). This notion is consistent with the extensive cerebrovascular amyloid deposition observed in patients with HCHWA-D. However, this interpretation does not explain the lack of mature fibrillar amyloid plaques and the accompanying neuronal degeneration in the brain parenchyma of patients with HCHWA-D (27–29).

In the present study, we provide evidence that the HCHWA-D mutation enhances the pathologic properties of Aβ. We have reported (18) that normal $A\beta_{1\text{-}42}$, but not the shorter $A\beta_{1\text{-}40}$, induced pathologic responses in cultured HLSM cells. In contrast, we show here that the shorter HCHWA-D mutant $A\beta_{1\text{-}40}$ induced even more robust pathologic responses in the cultured HLSM cells than normal $A\beta_{1\text{-}42}$.

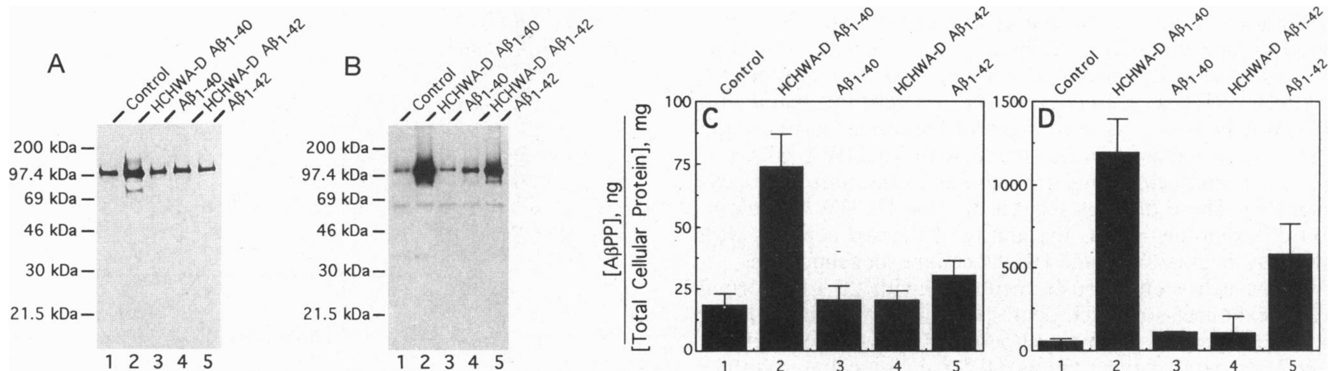

FIG. 3. Immunoblot analysis of secreted and cellular AβPP in cultured HLSM cells incubated with normal and HCHWA-D Aβ peptides. HLSM cells were incubated alone (lane 1) or with various Aβ peptides at 25 μM, including HCHWA-D $A\beta_{1\text{-}40}$ (lane 2), normal $A\beta_{1\text{-}40}$ (lane 3), HCHWA-D $A\beta_{1\text{-}42}$ (lane 4), and normal $A\beta_{1\text{-}42}$ (lane 5) for 6 days, and then 15-μl aliquots of each culture medium sample (*A*) or 3 μg of total protein from each cell lysate (*B*) was subjected to electrophoresis on nonreducing SDS/10% polyacrylamide gels and subsequently examined by immunoblot analysis with monoclonal antibody P2-1. The absolute levels of secreted (*C*) and cellular (*D*) AβPP in the HLSM cells incubated with the different Aβ peptides were determined by quantitative immunoblot analysis. Data for each peptide represents the mean ± SD of six or more determinations.

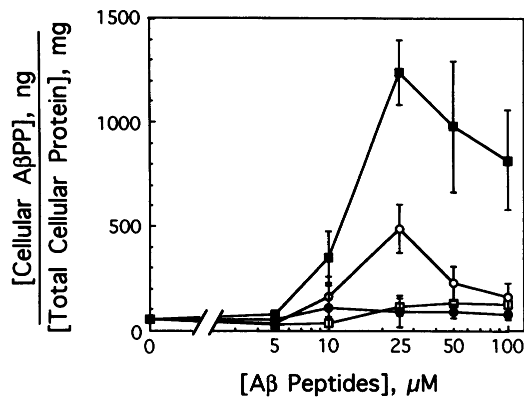

FIG. 4. HCHWA-D $A\beta_{1-40}$ and normal $A\beta_{1-42}$ cause increased levels of cellular $A\beta PP$ in cultured HLSM cells. HLSM cells were incubated with increasing concentrations of the normal and HCHWA-D $A\beta$ peptides for 6 days and the levels of cellular $A\beta PP$ were determined by quantitative immunoblot analysis. Each point represents the mean ± SD of six or more determinations. □, Normal $A\beta_{1-40}$; ■, HCHWA-D $A\beta_{1-40}$; ○, normal $A\beta_{1-42}$; ●, HCHWA-D $A\beta_{1-42}$.

For example, after 18 days of incubation, HCHWA-D $A\beta_{1-40}$ killed ≈90% of the HLSM cells compared to just ≈25% when the cells were incubated with normal $A\beta_{1-42}$ (Fig. 2). In addition, incubation of HLSM cells with HCHWA-D $A\beta_{1-40}$ caused an ≈25-fold increase in the levels of cellular $A\beta PP$ compared to an ≈10-fold increase when the cells were incubated with normal $A\beta_{1-42}$ (Figs. 3 and 4). This finding is consistent with the report by Rozemuller *et al.* (28) of increased accumulations of cerebrovascular $A\beta PP$ in patients with HCHWA-D compared to patients with AD. On the other hand, incubation of HLSM cells with normal $A\beta_{1-40}$ or HCHWA-D $A\beta_{1-42}$ resulted in no loss of cell viability (Fig. 2) or no appreciable increase in the levels of cellular $A\beta PP$ (Figs. 3 and 4). The more robust pathologic effects of HCHWA-D $A\beta_{1-40}$ on the cultured HLSM cells compared with normal $A\beta_{1-42}$ are consistent with the generally more severe cerebrovascular pathology observed in patients with HCHWA-D compared to patients with AD (5–8, 27–29).

It was intriguing that normal $A\beta_{1-42}$ induced pathologic responses in cultured HLSM cells whereas HCHWA-D $A\beta_{1-42}$ was devoid of this activity. It was observed that 25 μM HCHWA-D $A\beta_{1-42}$ aggregated in the culture medium of the HLSM cells within 1 h (Fig. 5). On the other hand, the same

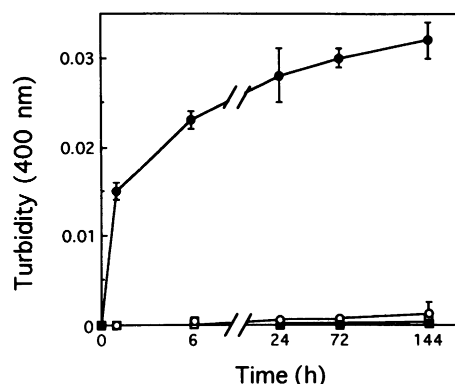

FIG. 5. Enhanced aggregation of HCHWA-D $A\beta_{1-42}$ in HLSM cell culture. HLSM cells were continuously incubated with the various $A\beta$ peptides at 25 μM. At designated times, aliquots of the medium were removed from the cultures and the turbidity of the samples was measured. Each point represents the mean ± SD of three determinations. □, Normal $A\beta_{1-40}$; ■, HCHWA-D $A\beta_{1-40}$; ○, normal $A\beta_{1-42}$; ●, HCHWA-D $A\beta_{1-42}$.

concentration of the shorter HCHWA-D $A\beta_{1-40}$ or the normal $A\beta$ peptides did not appreciably aggregate in the culture medium even after 6 days (Fig. 5). We recently reported that preaggregation of normal $A\beta_{1-42}$ nullified its ability to induce pathologic responses in cultured HLSM cells (19). This finding suggests that normal $A\beta_{1-42}$ must be in a soluble nonaggregated state to properly interact with the HLSM cells to elicit its pathologic effects. Since the HCHWA-D $A\beta_{1-42}$ aggregates in the HLSM cultures so rapidly, this likely negates its ability to properly interact with the HLSM cells in a manner to induce pathologic responses. In addition, this finding suggests that the HCHWA-D mutation enhances the ability of $A\beta_{1-42}$ to aggregate, consistent with other reports (25, 26), but does not noticeably enhance the aggregation of the shorter $A\beta_{1-40}$ peptide under the conditions examined.

Thus these studies demonstrate that the HCHWA-D mutation converts the normally nonpathologic $A\beta_{1-40}$ into a highly pathologic form of the peptide for cultured cerebrovascular smooth muscle cells. It is noteworthy that normal $A\beta_{1-40}$ and HCHWA-D $A\beta_{1-40}$ were reported to behave similarly with respect to inducing toxicity to cultured neurons (31). In addition, we have observed that cultured human endothelial cells and fibroblasts were resistant to the toxic effects of the both the normal and HCHWA-D mutant $A\beta$ peptides (data not presented). This suggests that the enhanced pathologic properties of HCHWA-D $A\beta_{1-40}$ are directed toward the cultured cerebrovascular smooth muscle cells not toward cultured neurons and other vascular cell types. This is consistent with the extensive cerebrovascular pathology and lack of neuronal pathology that is characteristic of HCHWA-D (27–29). Furthermore, studies with transfected cells have revealed that the majority of $A\beta$ produced is the shorter less-pathologic $A\beta_{1-40}$ (32). $A\beta_{1-42}$ may be the critical isoform for seeding the pathology of AD (33). Since this longer isoform of $A\beta$ is normally produced in small amounts, this may explain the decades necessary to manifest AD pathology. On the other hand, since the HCHWA-D mutation does not effect amyloidogenic processing of $A\beta PP$ (30), individuals with this disorder likely produce mostly $A\beta_{1-40}$. However, with the mutation this normally nonpathologic isoform of $A\beta$ now acquires highly pathologic properties for the cerebral vasculature. The present findings suggest that this functional alteration of $A\beta_{1-40}$ may contribute to the early and often severe cerebral amyloid angiopathy that is the hallmark of HCHWA-D.

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